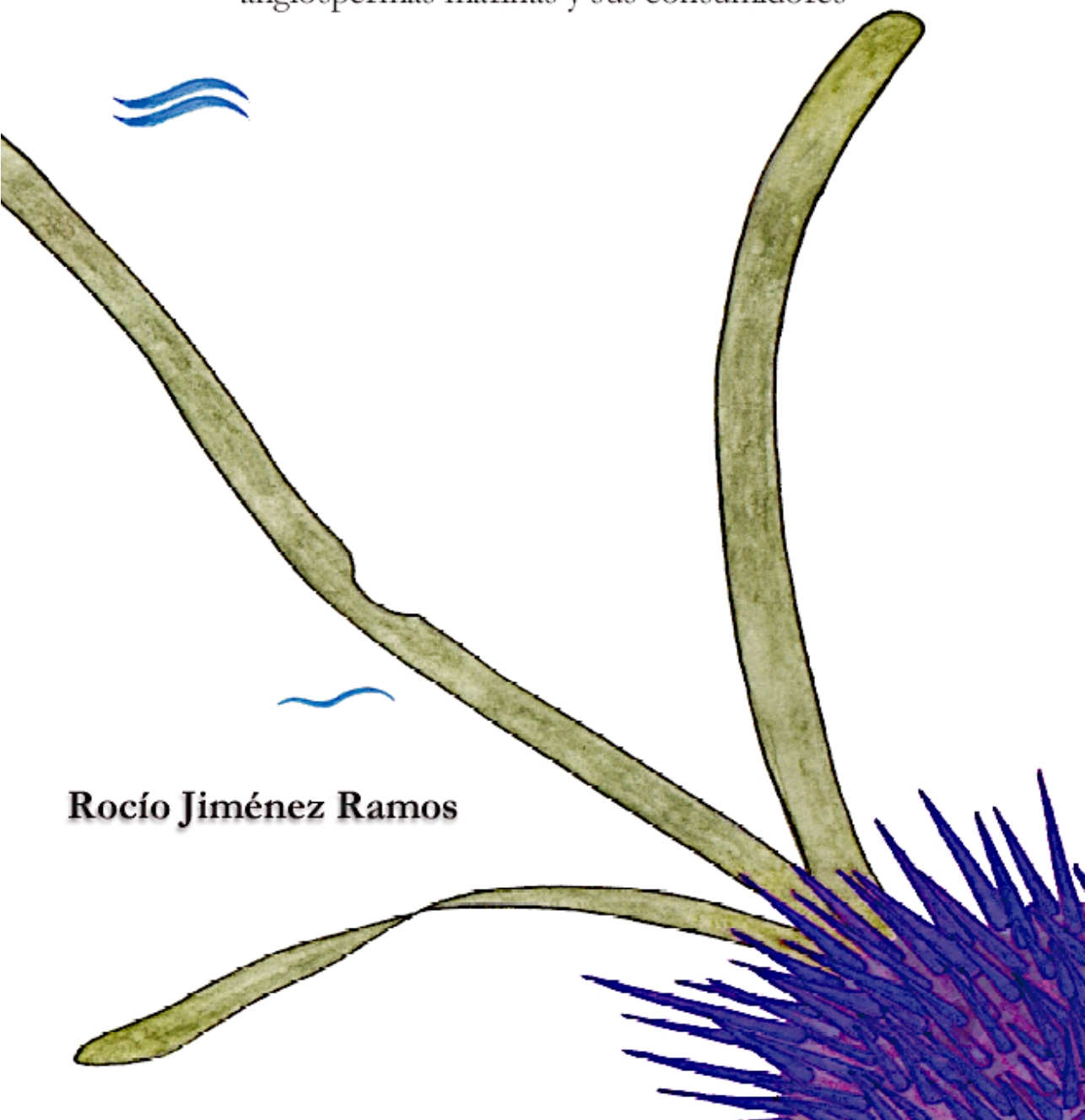
Influence of leaf traits, hydrodynamics and global change on seagrass-grazer interactions

Influencia de las propiedades foliares, la hidrodinámica y el cambio global en las interacciones entre angiospermas marinas y sus consumidores



Rocío Jiménez Ramos

R. Jiménez-Ramos was born on March 10, 1988 in Barcelona. Growing up by the Mediterranean sea and spent large summers with her family swimming under seagrass beds, in a fishing village called San Carles de la Ràpita (Tarragona), she fell in love with the ocean. This is why, after graduating high school in 2005, she decided to start a dual Bsc in Marine Science and Environmental Science at Cádiz University. As an undergraduate, she swam on the EDEA Team, and actively participated in the FAMAR volunteer program, which she is coordinating at present. During most of her university time (2006-2012), Rocío periodically worked as a Student Assistant for the department of Biology, accompanied by Dr. JJ Vergara and Dr. FG Brun. As a Student Assistant, she supported to several PhD colleagues to developed different seagrass experiments. Since then, she discovered the joy of walking on a muddy system, the pleasure of working under sea in a wet winter surrounded by laughter and the power of the team spirit. On August, 2011 she spent a full month to study the role of seagrass as a ecosystem engineers at The Royal Institute for Sea Research (NIOZ), as a Student Assistant. Additionally, she conducted her first personal experiment about seagrass eutrophication in Cádiz bay (Spain). After graduating in Marine Science, she received her Msc in Oceanography at Cádiz University. On the 1th of December, 2012, Rocío started working as a PhD-student looking at seagrass herbivory under hydrodynamics conditions and future scenarios of global change. Officially employed by Cádiz University, she also was stationed at the NIOZ (Yerseke, 2014) and at the Centro de Investigaciones Biológicas del Noroeste (CIBNOR) where she also collaborated in the Centro Interdisciplinario de Ciencias Marinas (CICIMAR) in Mexico (La Paz, 2015).

In addition to her research work, she is strongly committed to the transmission of knowledge and inspiring people to care the nature. Then, throughout her research career, she has been active in the dissemination of science.

Personal website: www.cmer.es

TESIS DOCTORAL

Influence of leaf traits, hydrodynamics and global change on seagrass-grazer interactions

Influencia de las propiedades foliares, la hidrodinámica y el cambio global en las interacciones entre angiospermas marinas y sus consumidores

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Memoria presentada por Rocío Jiménez Ramos para optar al Grado de Doctor por la Universidad de Cádiz

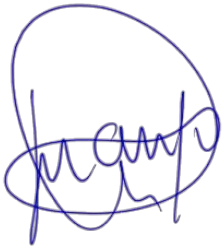
Fdo. Rocío Jiménez Ramos

Los directores: Dr. D. Juan José Vergara Oñate, catedrático de Ecología y Dr. D. Fernando G. Brun Murillo, Profesor Titular de Ecología de la Universidad de Cádiz.

CERTIFICAN:

Que la presente memoria titulada, "Influencia de las propiedades foliares, la hidrodinámica y el cambio global en las interacciones entre angiospermas marinas y sus consumidores", presentada por Rocío Jiménez Ramos, ha sido realizada bajo su dirección y autorizan su presentación y defensa, para optar al Grado de Doctor por la Universidad de Cádiz.

Y para que así conste y surta los efectos oportunos, firman los presentes en Puerto Real, a 27 de Septiembre de dos mil diecisiete.

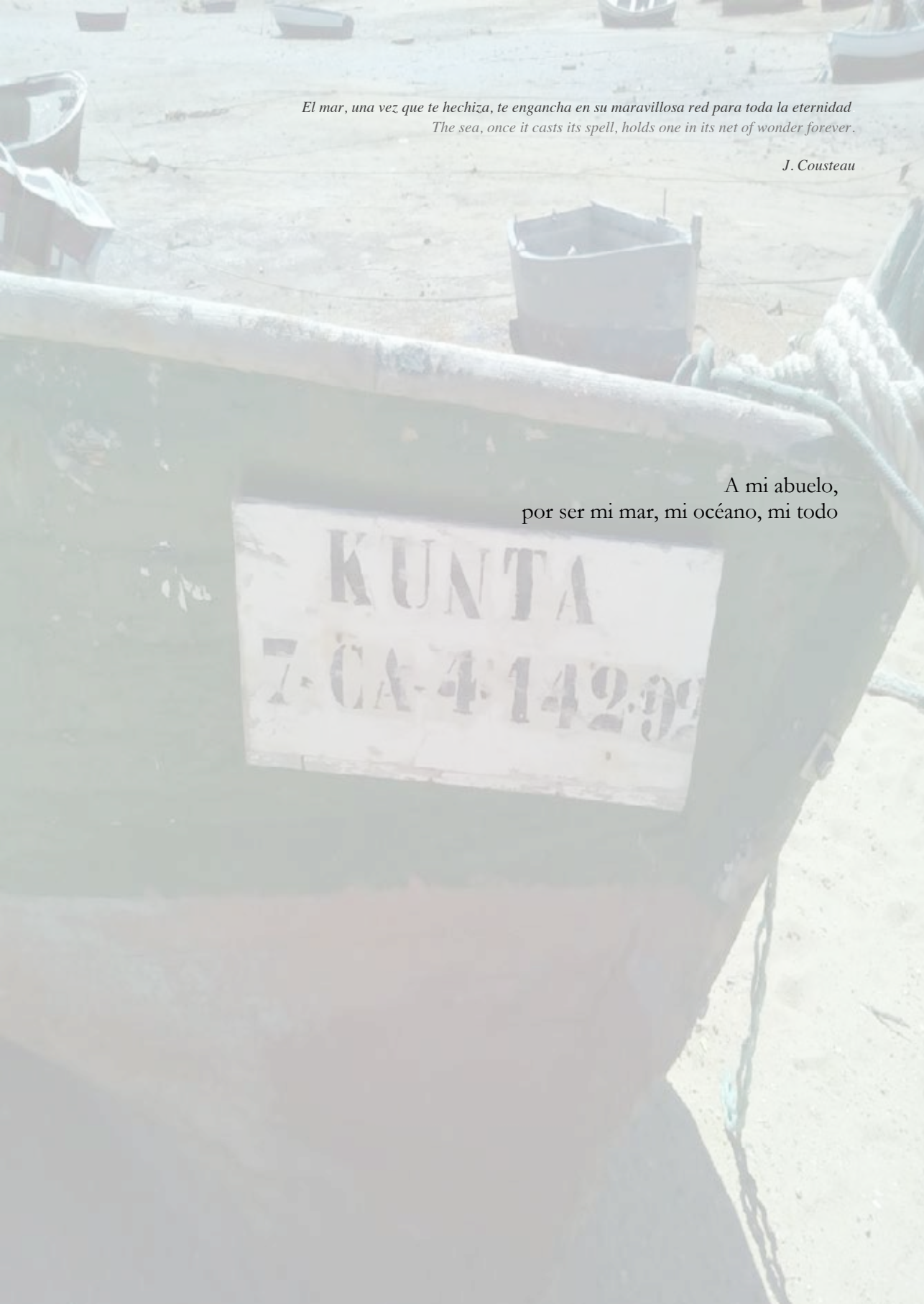


Prof. Dr. Juan. J. Vergara Oñate

Prof. Dr. Fernando G. Brun Murillo

Para la realización de esta Tesis doctoral, la doctoranda, Rocío Jiménez Ramos, ha disfrutado de una beca de posgrado del Programa de Formación de Personal Investigador (FPI) del Ministerio de Economía y Competitividad (BES-2012-055384) adscrita al proyecto de Plan Nacional de I+D+i "Retroalimentación y trade-offs en praderas de fanerógamas marinas: el coste de vivir en ecosistemas acuáticos" (SeaLive, Ministerio de Ciencia e Innovación, CTM2011-24482). Además, parte del trabajo presentado en esta memoria ha sido financiado por el proyecto de Excelencia concedido por la Junta de Andalucía "Producción y destino de carbono en las praderas marinas de la Bahía de Cádiz" (PRODESCA, P12-RNM-3020). La experimentación llevada a cabo en el Centro de Investigaciones Biológicas del Noroeste (CIBNOR) fue también parcialmente financiada por el "Programa de Ecología Pesquera" (PEP). Su actividad investigadora se ha realizado como miembro del grupo de investigación Estructura y Dinámica de Ecosistemas Acuáticos (PADI RNM-124), en el marco del Campus de Excelencia Internacional del Mar (CEIMAR).

Parte de la experimentación y análisis de la presente Tesis se ha llevado a cabo durante dos estancias de investigación financiadas por el programa FPI en las siguientes instituciones: Royal Netherlands Institute for Sea Research (Spatial Ecology Department, Yerseke, Holanda; bajo la supervisión del Dr. Tjeerd Bouma) y en Centro de Investigaciones Biológicas del Noroeste (Departamento de Ecología Pesquera, La Paz, México; bajo la supervisión del Dr. Eduardo Balart). Parte de los resultados presentados en esta memoria de Tesis Doctoral están actualmente publicados o bajo revisión en revistas internacionales indexadas (Journal of Ecology, PLoS ONE, Marine Ecology Progress Series, Estuarine, Coastal and Shelf Science), o se encuentran en preparación para su publicación. También parte de los resultados han sido presentados en congresos nacionales e internacionales (Seagrasses in Europe: Threats, Responses and Managament, Sania, China, 2014; ASLO Aquatic Sciences Meeting, Granada, España, 2015; VII Simposium Internacional sobre el Mar de Cortés, La Paz, México, 2016).



El mar, una vez que te hechiza, te engancha en su maravillosa red para toda la eternidad
The sea, once it casts its spell, holds one in its net of wonder forever.

J. Cousteau

A mi abuelo,
por ser mi mar, mi océano, mi todo

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madre por ser un ejemplo de generosidad, empatía y ternura. El objetivo de mi vida es llegar a ser como tu. A mi padre, por ser mi héroe. Eres la persona más especial, inteligente y leal que conozco. La persona que mejor me entiende, me conoce y me comprende. Gracias a los dos por dejar siempre todo atrás y acompañarme en cada aventura. Por enseñarme que lo más importante de la vida es el amor y la libertad.

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Rocío

CONTENTS

Abstract

Seagrass-grazer interactions play a fundamental role in ecological processes by regulating structure and functioning of plant communities. Within biotic connections, herbivory is a crucial process because it involves the matter and energy transference from primary producers to higher trophic levels, affecting the physical structure of the community and ecosystem productivity. Contrary to previous beliefs, new insights suggest that grazing may have an important impact on seagrass communities and that their grazers may be key to understand seagrass food webs. Thus, the contrasting response found in previous research may indicate that the susceptibility of seagrass species to be grazed may have a temporal and spatial component and even within a community, a preferential consumption of some species among others may exist depending on the palatability of their tissues. Therefore, the assessment of the strength of the seagrass-grazers links and those mechanisms that regulate this relationship, it is of crucial importance to increase our understanding on community and ecosystem structure and dynamics. In addition, it is well known that seagrasses are living under local and global threats, and consequently, there is a pressing need to include the global change factors (eutrophication, acidification and warming) into the current experimental approaches, in order to forecast the future responses of this fundamental community in the changing world.

This PhD Thesis is structured in 6 chapters to answer particular questions about seagrass-grazer interactions. In the first two chapters, the importance of hydrodynamics in the seagrass-grazer interactions was studied, and it demonstrated how this abiotic factor acts as a top-down regulator in the community. Moreover, seagrass acclimation to hydrodynamics modulates intraspecific seagrass traits, and then seagrass palatability, influencing the selection of leaves by consumers (chapters 2 and 3). On the other hand, eutrophication and the main global change factors (i.e. warming and acidification) influenced positively *Cymodocea nodosa* palatability through the alteration of leaf traits and by promoting the growth of epiphytes (chapters 4 and 5). In the last chapter, we looked at the seagrass responses to biomass loss promoted by herbivory, noting how the colonization states influences on the compensation responses to herbivory in the fast-growing tropical species such as *Halodule wrightii*.

The research presented in this PhD Thesis contributes to identify changes on seagrass palatability under different environmental conditions and global change factors. Particularly, this research displays how hydrodynamics modulate feeding behaviour of consumers and how indirect effects of eutrophication and global change enhance seagrass consumption by herbivores. In addition, this Thesis shows how seagrass colonization states affect to compensatory responses derived by herbivore damage.

Abstract

The importance of considering biotic processes in conservation and management plans is emphasized in order to enhance seagrass ecosystems resilience and to identify solutions to support the maintenance of key ecosystem services provided by seagrass meadows to our planet.

Resumen

Las interacciones entre planta-consumidor juegan un papel fundamental en los procesos ecológicos ya que regulan la estructura y el funcionamiento de las comunidades. Entre las diferentes relaciones bióticas, la herbivoría es un proceso clave porque da lugar a la transferencia de materia y energía de los productores primarios a niveles tróficos superiores, afectando la estructura física de la comunidad, la productividad y al propio ecosistema. A diferencia de los primeros estudios, en la actualidad se sugiere que el consumo sobre angiospermas marinas podría tener importantes impactos en sus comunidades y los consumidores pueden ser cruciales para entender las redes tróficas dentro de los ecosistemas dominados por estas plantas marinas. Los estudios basados en estas relaciones indican que la susceptibilidad de las especies de angiospermas marinas a ser consumidas puede tener un componente temporal y espacial e incluso dentro de una comunidad, puede existir un consumo preferencial de algunas especies frente a otras, dependiendo de la palatabilidad de sus tejidos. Por lo tanto, la evaluación de la fuerza de las interacciones entre planta-consumidor y los mecanismos que lo regulan es crucial para una mejor comprensión de las comunidades y la dinámica de los ecosistemas formados por angiospermas marinas. Además, está ampliamente demostrado que estas plantas marinas sufren un declive mundial y, como consecuencia, existe una necesidad urgente de incluir en los estudios aquellos factores relacionados con el cambio global (eutrofización, acidificación y calentamiento) para ahondar en la comprensión de la interacción entre fanerógamas marinas y sus consumidores.

Esta tesis está estructurada en 6 capítulos con cuestiones relacionadas con las interacciones planta-consumidor. En los dos primeros capítulos, se demostró la importancia de las condiciones ambientales en las interacciones planta-consumidor, considerando este factor ambiental como un regulador del control "top-down" en la comunidad. Además, la adaptación de las angiospermas marinas a las condiciones hidrodinámicas da lugar a cambios en las propiedades foliares, modificando su palatabilidad y que a su vez, influye en la selección de las hojas por parte de los consumidores (capítulos 2 y 3). Por otra parte, los principales factores relacionados con el cambio global (eutrofización, acidificación y calentamiento) influyeron positivamente en la palatabilidad de las hojas de *Cymodocea nodosa* a través de la variabilidad en sus propiedades foliares así como por la presencia de algas epífitas (capítulos 4 y 5). En el último capítulo, examinamos las respuestas de las plantas a la pérdida de biomasa por herbivoría, observando cómo las etapas de recuperación influyen en las respuestas de compensación a la herbivoría para especies tropicales de rápido crecimiento como *Halodule wrightii*.

En conclusión, la investigación presentada en esta tesis contribuye a identificar cambios en la palatabilidad de las angiospermas marinas bajo diferentes condiciones ambientales y factores de cambio global. En particular, esta investigación muestra cómo la hidrodinámica modula el comportamiento alimentario de los consumidores y cómo los efectos indirectos de la eutrofización y el cambio global aumentan el consumo de estas plantas marinas por los herbívoros. Además, esta tesis muestra cómo diferentes etapas de recuperación de angiospermas marinas afectan a las respuestas compensatorias derivadas del daño foliar por los herbívoros. Finalmente, se hace hincapié en la importancia de considerar a los procesos bióticos en los planes de conservación y manejo de los ecosistemas formados por angiospermas marinas, con el fin de mejorar su resiliencia e identificar nuevas soluciones para apoyar el mantenimiento de los servicios ecosistémicos que proporcionan estas plantas marinas a nuestro planeta.

Resum

Les interaccions entre planta-consumidor juguen un paper fonamental en els processos ecològics ja que regulen l'estructura i el funcionament de les comunitats. Entre les diferents relacions biòtiques, l'herbivorisme és un procés clau perquè dóna lloc a la transferència de matèria i energia dels productors primaris a nivells tròfics superiors, afectant l'estructura física de la comunitat, la productivitat i al propi ecosistema. A diferència dels primers estudies, treballs actuals suggereixen que el consum sobre les angiospermes marines podria tenir importants impactes en les seves comunitats i els consumidors poden ser crucials per entendre les xarxes tròfiques dins dels ecosistemes dominats per aquestes plantes marines. Els estudis basats en aquestes relacions indiquen que la susceptibilitat de les espècies d'angiospermes marines a ser consumides pot tenir un component temporal i espacial i fins i tot, dins d'una comunitat, pot existir un consum preferencial d'algunes espècies enfront d'altres, depenent de la palatabilitat dels seus teixits. Per tant, l'avaluació de la força de les interaccions entre planta-consumidor i els mecanismes que el regulen és crucial per a una millor comprensió de les comunitats i la dinàmica dels ecosistemes formats per angiospermes marines. A més, està àmpliament demostrat que aquestes plantes marines pateixen un declivi mundial i, com a conseqüència, hi ha una necessitat urgent d'incloure en els estudis aquells factors relacionats amb el canvi global (eutrofització, acidificació i escalfament) per aprofundir en la comprensió de la interacció entre angiospermes marines i els seus consumidors.

Aquesta Tesi Doctoral està estructurada en 6 capítols amb qüestions relacionades amb les interaccions planta-consumidor. En els dos primers capítols, es va demostrar la importància de la hidrodinàmica en les interaccions planta-consumidor, considerant aquest factor ambiental com un regulador del control "top-down" en la comunitat. A més, l'adaptació de les angiospermes marines a les condicions hidrodinàmiques dóna lloc a canvis en les propietats foliars, modificant la seva palatabilitat i que al seu torn, influeix en la selecció de les fulles per part dels consumidors (capítols 2 i 3). D'altra banda, els principals factors relacionats amb el canvi global (eutrofització, acidificació i escalfament) van influir positivament en la palatabilitat d les fulles de *Cymodocea nodosa* a través de la variabilitat en les seves propietats foliars així com per la presència d'algues epífites (capítols 4 i 5). En l'últim capítol, examinem les respostes de les plantes a la pèrdua de biomassa per l´herbivorisme, observant com les etapes de recuperació influeixen en les respostes de compensació a la herbivoria per a espècies tropicals de ràpid creixement com *Halodule wrightii*.

En conclusió, la recerca presentada en aquesta Tesi contribueix a identificar canvis en la palatabilitat de les angiospermes marines sota diferents condicions ambientals i factors de canvi global. En particular, aquesta investigació mostra com la hidrodinàmica modula el

comportament alimentari dels consumidors i com els efectes indirectes de l'eutrofització i el canvi global augmenten el consum d'aquestes plantes marines pels herbívors. A més, aquesta tesi mostra com diferents etapes de recuperació d'angiospermes marines afecten a les respostes compensatòries derivades del dany foliar pels herbívors. Finalment, es remarca la importància de considerar els processos biòtics en els plans de conservació i maneig dels ecosistemes formats per aquestes plantes, per tal de millorar la seva resiliència i identificar noves solucions per donar suport al manteniment dels serveis ecosistèmics que proporcionen aquestes plantes marines al nostre planeta.

GENERAL INTRODUCTION

Los mejores maestros son aquellos que te muestran donde mirar pero no te dicen qué ver.
The best teachers are those who show you where to look, but don't tell you what to see.

Alexandra K. Trenford

Why plant-grazer interactions? The role of herbivory

Biotic interactions between plants-grazer play a fundamental role in nature by regulating the structure and functioning of communities (Bardgett and Wardle 2010). The variation in the influence of consumers impact on resource availability has been a primary objective in the ecology of communities (Hairston et al. 1960, Gruner et al. 2008), showing that the top-down control is spatially and temporally variable, and it is a factor explaining global biodiversity gradients (Moles et al. 2011). Within plant-grazer interactions, herbivory is the process that focus on the consumption of plants by herbivorous and it is considered as a key process in the functioning of the ecosystems, since is one of the locomotives of natural selection (Schowalter 1981). In every piece of wood, bark, meadow and forest, plants use mechanical and chemical tools to survive and reproduce in the company of herbivores that can consume them. Then, herbivory involves the transference of matter and energy from primary producers to higher trophic levels, affecting the physical structure of the community and the ecosystem, and the habitat productivity (McNaughton 1984, Cyr and Face 1993). In order to understand the role of herbivores in the ecosystems, the impact they have on primary producers has been quantified, and then try to relate these impacts with those mechanisms that explain the variation in the strength of biological interactions. This includes primary and secondary production rates, nutritional quality, resistance and tolerance to herbivory by primary producers, feed behaviour of herbivores, etc (McNaughton et al. 1996, Cebrián and Lartigue 2004).

"... herbivory is the process that focus on the consumption of plants by herbivorous consumers and it is considered as a key process in the functioning of ecosystems..."

Deepen on these biotic interactions in coastal areas are entirely relevant, since coast is the part of the ocean most affected by its proximity to the land (Hinrichsen 1998). Coastal zone presents a large variety of ecosystems in both terrestrial and marine parts (Martínez et al. 2007) which are exposed to a range of anthropogenic threats (e.g. coastal urbanization, habitat fragmentation, fishing and aquaculture, etc.) (Serviere-Zaragoza et al. 2012, Olafsson 2016). Within the marine part, coastal vegetated habitats (including seagrass meadows, macroalgae, mangroves and salt marshes) occupy only a limited surface on a global scale (<2% of the ocean surface) but have some of the highest rates of primary production (Duarte and Cebrián 1996, Nellemann et al. 2009). Especially, seagrass ecosystems are considered the most

productive ecosystems worldwide but the most threatened as well (Waycott et al. 2009). The mean net community production of seagrasses (NCP ca. 120 g C m^{-2} y^{-1}) (Duarte et al. 2010), represents globally between ca. 36 – 72 Tg C y^{-1} considering the range estimated of seagrass cover area (between 300,000 to 600,000 km^2) (Hemminga and Duarte 2000). The fate of this production could be (1) around 44% exported either particulate and dissolved form (2) around 16% sequestered in the sediment contributing to the marine C burial (i.e. blue carbon) and (3) around 18% is consumed by herbivores (Duarte and Cebrián 1996). However, the global estimates of seagrass herbivory rates in the literature are widely variable (from <10% up to 100% of total production, Heck and Valentine 2006) and most of the existing comparisons are not extensive at latitudinal level (Cebrián et al. 1996a, Cebrián 2002). Thus, further efforts are needed to elucidate if there are patterns in how herbivory varies within and across seagrass communities, and to identify the controls and consequences of such variability. Hence, seagrass ecosystems require an in-depth research regarding their interactions with grazers, the environment and the response to the global change threats (Figure 1).

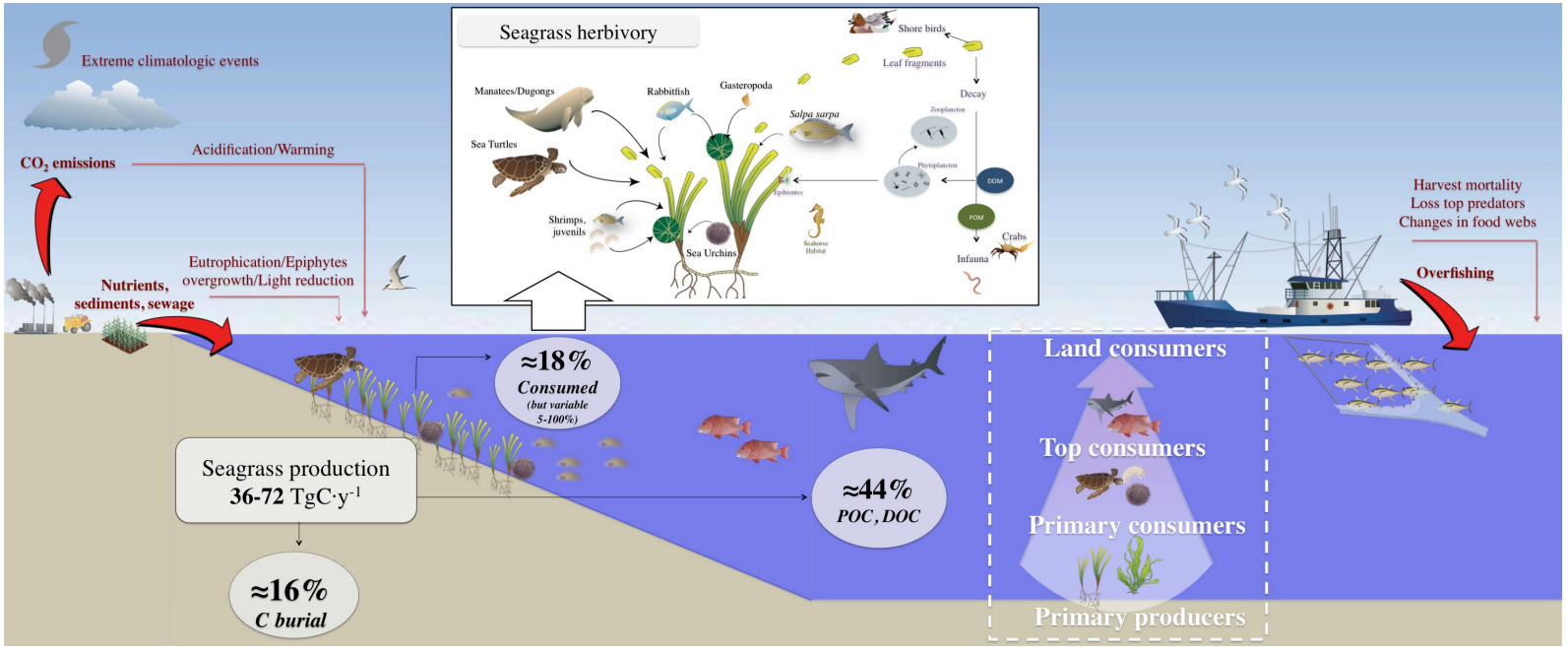


Figure 1. Schematic illustration of simplified biotic interactions (white letters) and negative impact (red letters) to seagrass ecosystems. Schematic seagrass herbivory process on superindexed square.

Herbivory on seagrass ecosystems: a paradigm shift

Seagrasses are the only group of flowering plants that perform life cycle in marine environments (den Hartog 1970). They are composed of repeating units (ramets) that exhibit clonal growth, being rhizomatous (they have stems extend in horizontally below the sediment surface) and modular plants (Hemminga and Duarte 2000). Seagrasses develop flowers, fruits and produce seeds (Ackerman 2006). Moreover, seagrasses have true roots and internal gaseous and nutrient transport systems (den Hartog and Kuo 2006). Currently, the functional definition for seagrass plants encompasses around 60 species. Although the gross majority of species are considered common, 3 are considered endangered and 10 are at elevated risk of extinction (Short et al. 2011).

Sometimes, seagrass beds have been considered like mucky meadows in coasts, since they lack the charisma of coral reefs (Duarte et al. 2008). However, seagrass meadows are one of the most productive ecosystems on earth and are important ecological and thus economical components of coastal zones worldwide (Costanza et al. 1997, Waycott et al. 2009). Nevertheless, despite its ecological importance, the new knowledge about seagrasses comes at a crucial time as 7% of seagrass beds disappear every year (Pennisi 2012). Because of its unprecedented global loss rate, the interest in understanding the factors that contribute to preserve healthy seagrass meadows has simulated (Duarte 2002), even leading to the estimation of the economical value of these ecosystems (Costanza et al. 1997, Green and Short 2004, Unsworth et al. 2010). Valuable ecosystem functions generally associated with seagrass habitats include nutrient recycling since they act like biofilters by stripping nutrients and other contaminants from the water through foliar uptake (Van Katwijk et al. 1997, Villazán, Pedersen, et al. 2013); improve water transparency since they enhance the settling of suspended particles; sediment stabilization with their rhizomes, roots and leaves contributing to shoreline protection (Orth et al. 2006, Peralta et al. 2008); acting as a hotspot of carbon sequestration and carbon sink in the biosphere (Duarte et al. 2005, Kennedy et al. 2010, Egea et al., 2017); provision of optimal habitat for growth, survival and reproduction of a diverse array of vertebrate and invertebrate taxa (Duffy 2006; Duarte et al., 2008; González-Ortiz et al. 2014).

"... seagrass beds have been considered like mucky meadows in coasts but are one of the most productive ecosystems on earth and are important ecological and thus economic components of coastal zones worldwide".

One of the most frequently cited indicators of seagrass habitat value, and one that is very often argued for its conservation and restoration, is its 'nursery function'. In this way, the growing understanding that seagrass meadows, as a foundation species, serve as "nursery habitats" for a variety of economically important finfishes and shellfishes (see Heck et al. 2003; Castañeda-Fernández de Lara et al. 2015) (Figure 2), coupled with the global decline of seagrasses during the last decades (Short and Wyllie-Echeverria 1996), has stimulated the interest of seagrass species and the organisms that inhabit seagrass communities (Valentine and Heck 1999, Duffy 2006, Stachowicz et al. 2007).

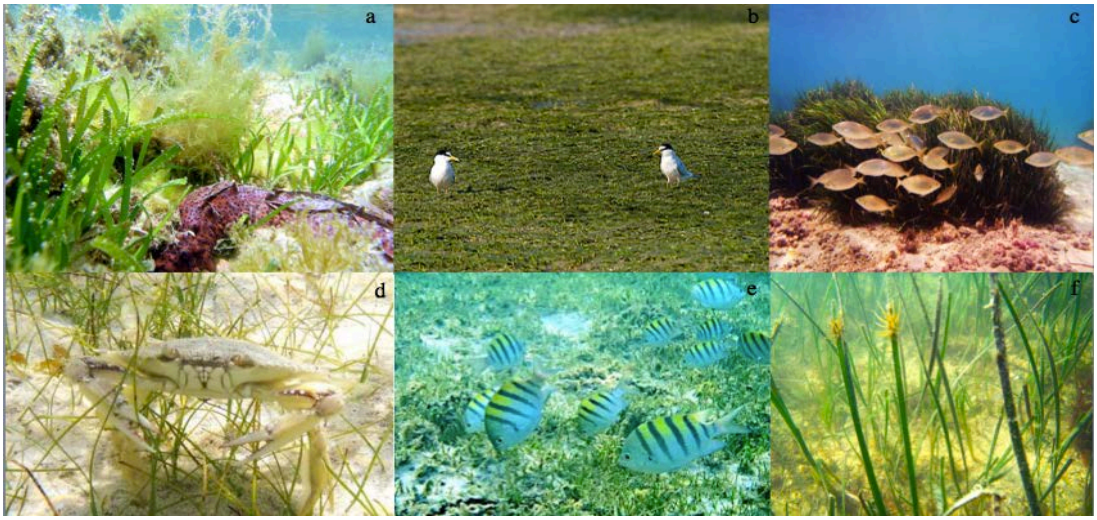


Figure 2. Seagrass meadows and some examples of associated fauna. a) *Holoturia* sp. in *Cymodocea nodosa* meadow; b) *Sternula albifrons* in *Zostera noltei* meadows; c) *Salpa sarpa* in *Posidonia oceanica* meadows; d) *Brachyura* sp. in *Halodule wrightii* meadow; e) Mugelin in *H. wrightii* meadow; f) Sea anemones in *C. nodosa* meadow (a, b photographs by Fernando G. Brun; c photograph by Ricardo Bermejo; d, e photographs by Claudia J. Pérez-Estrada; f photographs by Luis G. Egea).

Notwithstanding seagrass ecosystems are considered as an oasis of biodiversity, little attention was paid to herbivory process in past studies. This is due to the accepted idea by earlier works, which contemplated a minimal seagrass consumption (Kikuchi and Peres 1977, Thayer et al. 1984, Zieman et al. 1989). This conclusion was based on the observed presence of the abundant detrital seagrass leaves on some sediments and shorelines (Fenchel 1977). Moreover, it should also be noted that the grazing paradigm for seagrass meadows is similar to terrestrial ecologists (e.g., Lowman 1984, McNaughton 1985, Karban and Baldwin 1997), who underlined the importance of detrital pathway over that of the grazing pathway in terrestrial food webs. Hence, while the importance of grazing in algal-dominated communities is well documented (Strong 1992, Hay 1997), the premise that the ingestion of living seagrass

biomass is unusual and inconsequential has been largely accepted. Consequently, the herbivory rates collected in early literature have been generally modest, ranging from 10–15% of the seagrass production removed (den Hartog 1970, Ott 1981, Thayer et al. 1984) However, former evidences suggested that this was an oversimplification of the importance of grazing in both modern-day and historical seagrass food webs (see review by Valentine and Duffy 2006 and reference therein). In tropical and subtropical latitudes herbivores may consume up to 5–50% and 5–90% of the leaf production of *Thalassia testudinum* (Greenway 1976, Zieman et al. 1979, Valentine and Heck 1991) and *T. hemprichii* (Hattori et al. 1985, Mukai and Nokima 1985, Klumpp et al. 1993) respectively. Other pioneering observations at that time emphasized the influence of herbivory by fishes (Mariani and Alcoverro, 1999). On the other hand, studies on seagrass herbivory carried out in temperate locations also pointed to a remarkable variability, as shown by reports of herbivory on *Zostera noltii* (15–65% of leaf production, Jacobs et al. 1981, Vermaat and Verhagen 1996), *Posidonia oceanica* (2–37%, Ott and Maurer 1977, Kirkman and Young 1981, Velimirov 1984, Cebrián et al. 1996a, Prado et al. 2007) and *Cymodocea nodosa* (1–45%, Cebrián et al. 1996b). Therefore, studying the determination of patterns in seagrass herbivory and the interactions among grazers will lead to greater understanding of the magnitude of herbivory in these ecosystems and would allow to set limits to the general role of seagrasses as a food resource for herbivores (Cebrián and Duarte 1998).

Seagrass food webs: interaction with associated grazers

Herbivores often greatly influence the productivity and abundance of plants in aquatic and marine environments (e.g. Porter 1973, Lynch and Shapiro 1981, Mallin and Paerl 1984). Therefore, plants have to adapt to such stress and evolve, in part, under the pressure of its interactions with their main grazers (Barnard and Frankel 1964, Marck and Thompson 1982, McNaughton 1984, Coughenour 1985). Despite seagrasses developed independently of terrestrial grasses (Les et al. 1997), they possess many of the same traits that are considered to be grazer-derived adaptations (Valentine and Duffy 2006). Among these traits are: clonal propagation and the resultant physiological integration of ramets; the possession of largely inaccessible belowground basal meristems and branching rhizomes; abundance of small deciduous shoots and the ability to rapidly regenerate defoliated tissues (Valentine and Heck 1999). In addition, it is believed that the diversity of species in tropical environments is due to the grazing activity throughout the life-history of these species. With these premises in mind, a series of experiments on seagrass herbivory were performed by scientific community and the results showed how large vertebrates like dugongs or green turtles exerted selective pressure on foliar tissues (Bjorndal 1980, Preen 1995). Hence, contrary to previous beliefs, these findings suggested that grazing could have an important

impact on the species composition of seagrass communities, and that grazers are a key factor to understand seagrass food webs.

"… seagrass possess many of the same terrestrial plant traits considered to be grazer-derived adaptations…"

Different species and number of herbivores characterize the seagrass food webs and feed of their production (Figure 3). This means that de transference of energy from seagrass meadows to higher trophic levels is based on diverse players with different magnitudes. Even mobile herbivores such as fishes are considered as a mobile link by connecting shallow and deep areas of seagrass meadows (Pagès et al. 2013). In tropical settings, fishes and larger vertebrates are considered dominant herbivores (Thayer et al. 1984, Aragones and Marsh 2000, Lee et al. 2015) although they are also found as part of the diet of invertebrates (Serviere-Zaragoza et al. 1998, Mazariegos-Villareal et al. 2013). In lower latitudes, studies have shown that a number of sea urchins and herbivorous fish species ingest large amounts of aboveground seagrass biomass (e.g. Lawrence 1975, Valentine and Heck 1991, Klumpp et al. 1993, Alcoverro and Mariani 2002, Tomas et al. 2005, Prado et al. 2007). Especially in Mediterranean and Atlantic sea, the sea urchin *Paracentrotus lividus* and the fish *Salpa sarpa* are the two main herbivores, even were responsible of overgrazing events (Boudouresque and Verlaque 2001, Tomas et al. 2005a, Pinna et al. 2009). The large assemblages of sea urchins generate negative pressure in the systems formed by marine angiosperms and completely deplete seagrass areas of thousands of square meters (Valentine and Heck 1991, Alcoverro and Mariani 2002, Eklöf et al. 2008, Goldenberg and Erzini 2014). These phenomena underline the importance of studying the preferences of herbivores for the seagrass species and/or kind of leaf in order to understand the complex relationships that bind herbivores and seagrass, highlighting the role of grazing in influencing seagrass abundance and distribution.

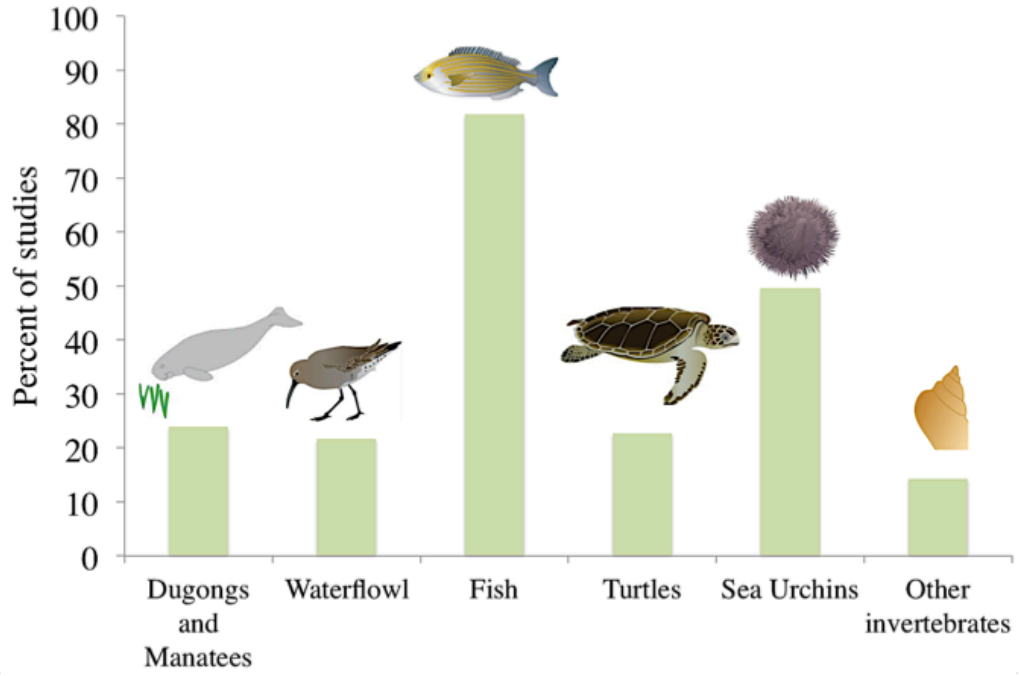


Figure 3. Frequency distribution of studies carried out on various types of seagrass herbivores from 2005 (after Valiela, 2005; reviewed from Valentine and Heck, 2006) to 2017.

Influence of leaf traits in feeding decisions by seagrass grazers

Different leaf traits influence food selection by seagrass consumers, especially those related to anti-herbivore defenses, by trying to reduce the attractiveness of tissues as food source, that is by altering their palatability. Palatability in this sense should be understood as any property of a plant that can modify their acceptability by herbivores (i.e. vulnerability to be consumed) (Heady and Child 1994). These traits include the reduction of plant quality as food (i.e. nutritional traits), the synthesis of chemical deterrents (i.e. chemical traits)or the reinforcement of tissues (i.e. mechanical and morhological traits) (Zapata and McMillan 1979, McMillan et al. 1980, Vergés et al. 2007a, b, Prado and Heck 2011).

a) Nutritional traits

The nutritional properties of the leaves (often expressed as leaf nitrogen content) have been shown to play a central role as a determinant in the seagrass palatability, since nitrogen (N) is a limiting resource for many herbivorous organisms (Mattson 1980). It has been demonstrated that lower C:N ratio supported higher herbivory pressure, since plants are

more palatable to herbivores (Cebrián and Duarte 1994). In general, a number of studies have found positive correlations between the N content and the rate of consumption of certain species of macroalgae and seagrasses (Bjorndal 1980, Zieman et al. 1984, Preen and Marsh 1995, Goecker et al. 2005, Cebrián et al. 2009). Consequently, these observations have led to conclude that, as has been suggested for many herbivorous insects and mammals, herbivore feeding on seagrasses is determined largely by nitrogen availability in tissues, rather than by others leaf properties. Hence, many researches have hypothesized that low leaf nitrogen concentration is a form of plant defense against herbivory (Feeny 1970, Augner 1995). Moreover, N content is variable among species (Duarte et al 1990; Romero et al. 2006), and even displays spatial-temporal differences in a single species (De los Santos et al. 2013; Villazán et al. 2016). Therefore, the contribution of N to the plant palatability may be subjected to inter- and intra-species variability in addition to a spatial-temporal dependence according to environmental conditions.

b) Biomechanical traits

Biomechanical traits such as leaf toughness have been commonly reported as an herbivore deterrent strategy in terrestrial plants because these properties limit the ability of the herbivores to shear or to tear the leaves for their intake (Lucas 2000, Sanson 2006). However, it has been investigated only recently in seagrass (Vergés et al. 2011, De los Santos et al. 2012, 2013). These recent publications suggest biomechanical traits may be considered as an important leaf trait controlling seagrass herbivory. Moreover, there are also studies showing differences within and among species in addition to a spatial-temporal dependence in biochemical leaf traits according to environmental conditions (La Nafie et al. 2013, De los Santos et al. 2012, 2013, Martínez-Crego et al. 2016). In particular the case of hydrodynamics, plant adjustments involving changes in morphometric and structural traits have been demonstrated in seagrasses as an acclimation process (Schanz and Asmus, 2003, Peralta et al. 2006).

c) Morphological traits

Length, width and thickness of leaves encompass the morphological traits. Seagrass come in a great variety of sizes and shapes, from the largest seagrass with strap-like leaves (*Enhalus acoroides*, den Hartog 1970) to small pairs oval leaves (e.g. *Halophila* sp.). Especially, hydrodynamics modulate seagrass leaf morphometry even within species (De Los Santos et al. 2013), which could influence on plant susceptibility to herbivores. In fact, Martínez-Crego et al. (2016) showed a positive correlation between thinner leaves and consumption rates.

d) Structural traits

Structural properties could be related with carbon or fiber content and confer low palatability to seagrasses due to the difficulty to bite this tissue and/or their reduced digestibility (Wright and Vicent 1996). Some publications on traits mediating feeding choices of seagrass consumers suggest that structural traits of seagrass leaves may play an important role in regulating the plant-herbivore interactions in seagrass ecosystems, the same as suggested for terrestrial and salt marsh plants (Grime 1996, Pennings et al. 1998, Mariani and Alcoverro 1999, Prado and Heck 2011).

e) Chemical traits

Levels of chemical defenses have been commonly pointed out as the most important determinants of food choice by most herbivores in seaweed communities (Bolser and Hay 1996, Prusak et al. 2005). Main inducible chemical traits include a wide range of defensive proteins and secondary metabolites that have toxic, deterrent, and/or digestion-reducing effects on herbivores (Bennett and Wallsgrove 1994, Kessler and Baldwin 2002). In particular, phenolic compounds with well-known defensive properties in both terrestrial plants and seaweeds (Bennett and Wallsgrove 1994, Amsler and Fairhead 2006) are also widely abundant in seagrasses (Zapata and McMillan 1979), but their function in these marine plants remains largely uninvestigated (Serviere-Zaragoza et al. 2002, Vergés et al. 2007a, Martínez-Crego et al. 2016). Moreover, recent studies have found a variability on chemical compounds under future scenarios of global change (e.g. Arnold et al. 2012; Hernán et al. 2017), which may influence on seagrass palatability and then, in herbivory patterns.

Although most herbivores select food with high nutritional content or low levels of defenses (Goecker et al. 2005; Cebrián et al. 2009; Vergés et al. 2007b, Tomas et al. 2011a), we know little about how these multiple traits interact and influence feeding preferences (Hay et al. 1994). Some studies actually found a positive correlation between seagrass leaf N content and preference by herbivores (i.e. Bjorndal 1980, Zieman et al. 1984, McGlathery 1995, Goecker et al. 2005), but other studies highlighted the importance of structural or chemical traits mediating seagrass grazing (Mariani and Alcoverro 1999, Vergés et al. 2007b, Prado and Heck, 2011, White et al. 2011). Moreover, few studies have been involved the role of chemical traits, since results cannot be generalized (e.g. Harrison 1982, Paul et al. 1990, Vergés et al. 2007a).

"Palatability should be understood as any property of a plant that can modify their acceptability by herbivores (i.e. vulnerability to be consumed)".

These contrasting findings on traits mediating feeding choice by seagrass consumers may indicate that herbivore preference over seagrass tissues cannot be only described by a single trait, but by the combination of all those leaf traits contributing to palatability. Therefore, the susceptibility of seagrass species to be grazed may have a temporal and spatial component and even within a community, a preferential consumption of some species among others may exist depending on the palatability of their tissues (i.e. inter-specific differences). Otherwise, the contribution of such mechanisms to palatability probably also depends on environmental conditions, such as hydrodynamics (i.e. intra-specific differences).

Effects of hydrodynamic conditions on seagrass-grazer interactions

Besides herbivory, seagrasses are adapted to marine life and as a consequence are exposed to constant risk of structural damage due to abiotic factors, such as waves and currents. It is therefore essential for the plants to protect against structural failure (Read and Stokes 2006 and references therein). Leaf mechanical resistance can be assessed by their fracture properties such as toughness and strength, which are traits describing how and under what conditions the leaf will break (Aranwela et al. 1999). This increase in the mechanical resistance of leaves is achieved by leaf toughening since plants invest in cell wall material and fibers, in addition to rise on width and thickness (Lucas 2000; De Los Santos et al. 2013). Thereby, the adaptation to the hydrodynamic conditions of the medium could have consequences in the selection of leaves by the seagrass consumers. For instance, leaf toughening has been reported as an effective defense against herbivores in terrestrial plants since it limits the ability of herbivores to cut or tear leaves for ingestion (Grime 1996, Díaz et al. 2001, Sanson et al. 2001, Siska et al. 2002, Sanson 2006). Regarding seagrasses, although authors showed that structural properties such as carbon and fibers content modulated the decisions made by herbivores and, therefore, hypothesized about the importance of biomechanical properties in leaf selection by herbivores (De los Santos et al. 2012, Tomas et al. 2015), this consideration has been poorly studied with direct measurements in feeding choices experiments (e.g. Martínez-Crego et al. 2016).

"Seagrass acclimation to the hydrodynamic conditions could have consequences in the selection of leaves by seagrass consumers"

In addition, seagrasses are important ecosystem engineers, which can change the physical environment through their physical structures (Bouma et al. 2005, Jones et al. 2010, González-Ortiz, et al. 2014a). Such habitat modification can result in positive feedbacks, stabilizing seagrass meadows (Fonseca and Fisher 1986, Siebert and Branch 2005, 2007, Goshima and Peterson 2012) as well as having either a positive (e.g. facilitation) or negative effects on the survival of associated species (Bertness and Leonard 1997, González-Ortiz et al. 2016). Numerous studies over the last decades have explored how physical and biological habitat modification promoted by seagrass meadows affects the occurrence of filter- feeding infauna (e.g. distribution, survival, growth, etc.), which constitutes an ecologically and economically important group of marine species (Wilson 1991, Dang et al. 2010, González-Ortiz et al. 2014a, González-Ortiz et al. 2014b). Nevertheless, the effect of water movement on the complex seagrass-grazer interaction has rarely been considered (but see Schanz et al. 2002). The numerous studies carried out by Koehl's group revealed how hydrodynamics affect the swimming capacity, feeding and recruitment of different marine organisms (Koehl 1984, Denny et al. 1985, Dickinson et al. 2000, Koehl and Reidenbach 2010, Pepper et al. 2015). Therefore, the presence of currents should affect the physics of seagrass consumers, especially small decapods or amphipods, called mesograzers, which are key components in trophic cascades on seagrass meadows (Moksnes et al. 2008, Best and Stachowicz 2012).

Effects of global change factors on seagrass-grazer interactions

The role of global stressors in altering marine ecosystems has received growing attention over the past decade. Seagrass communities are under threats from co-occurring local (e.g. eutrophication) and global stressors (e.g., ocean acidification, rising temperatures), yet understanding the interactive impacts of multiple stressors in ecosystem function is far from being accomplished. Abiotic changes can modify plant–herbivore interactions, both directly by affecting the physiology of the grazers, and also indirectly by altering the leaf traits of plants, and thus changing the leaf palatability (Cruz-Rivera and Hay 2000, O'Connor 2009, Tomas et al. 2015).

The most common mechanism responsible for seagrass decline in shallow coastal areas is eutrophication, which affects seagrass ecosystem in several ways. On the one hand, nutrient increase influence on different components of seagrass community (Jiménez-Ramos

et al. 2017a) stimulating algal and epiphyte overgrowth and thereby strongly reduces light availability for seagrasses (Hughes et al. 2004, Burkholder et al. 2007). On the other hand, eutrophication stimulates seagrass consumption by herbivores, probably through an increase in the nutritional quality of its food sources (Cebrián and Lartigue 2004), which can lead to a significant reductions in photosynthetic biomass and consequently to seagrass mortality (Invers et al. 2004, Villazán et al. 2013). On the other hand, it has been postulated that although internal contents in N is a relevant factor mediating feeding preferences by marine herbivores (Cebrián, 2009), the epiphytic load on leaves may strongly influence herbivore consumption on seagrass ecosystems (Marco-Méndez et al. 2015). However, as the nutritional quality, in other occasions its influence in seagrass consumption is negligible (Lee et al. 2015). Given that several factors (e.g. leaf traits, epiphyte presence, hydrodynamics) could be involved in the complex seagrass-grazers interactions, further studies are required to combine experimental approaches integrating them.

"Global change factors can modify plant–herbivore interactions, both directly by affecting the physiology of the grazers, and indirectly by altering the leaf traits of plants, and thus changing the leaf palatability"

On the other hand, human activity is increasing the concentration of CO_2 in the atmosphere at the rate of 0.4% yr^{-1} and it is expected to double from preindustrial levels by the middle of this century (Houghton 2001). Marine waters have absorbed approximately 30 per cent of CO_2 emissions and many marine species are already being forced to cope with increasing ocean acidification. Evidences in terrestrial systems indicate that increased CO_2 can decrease plant susceptibility to herbivory by enhancing resistance traits. Indeed, plants commonly respond to increased CO_2 by decreasing leaf nutritional quality (e.g., decreasing N and increasing C:N) and enhancing the production of chemical defenses (e.g., phenols), and these changes in plant traits may have consistent effects on herbivore feeding rates (Stiling and Cornelissen 2007). In seagrass literature, there is a lack of uniformity in the response of seagrasses to CO_2 changes, indicating either no change or a decrease in nutrients content (Arnold et al. 2014), and no change or a decrease in phenolic compounds (e.g. Arnold et al. 2012, Martínez-Crego et al. 2014). Moreover, different responses have been found among species of consumers. For example, while Tomas et al. (2015) did not detect chemical changes associated with CO_2, a differential responses of sea urchins (negatively) and isopods (positively) to plant grown under high CO_2 levels were reported. Meanwhile, a positive response of sea urchin to seedlings exposure to high CO_2 levels were found by Hernán et al. (2016). Moreover, scaling up to higher trophic levels, ocean acidification even can act as a diver

of seagrass community simplification limiting trophic propagation to predator populations (Vizzini et al. 2017).

Otherwise, global warming is emerging as a major threat to ecosystems worldwide (IPCC 2007). Mean global sea-surface temperatures have increased by ~0.8 °C over the last century (Levitus et al. 2001, Hansen et al. 2006) and, by the end of this century, is projected to increase by ~3-4 °C (Meehl et al. 2007). However, few studies have addressed the effect of warming on species interactions in the ocean (Wernberg et al. 2012). Previous research into the effects of warming on seagrasses is largely limited to physiological and metabolic responses, with an indication that photosynthetic capacity is reduced at high temperatures (Campbell et al. 2006, Olsen et al. 2012), or growth rate is decreased with warming (Olsen et al. 2012). Thus, warming temperatures may influence the strength of the top-down control by affecting the abundance and distribution of producers or herbivores (Wernberg et al. 2011), the consumption rates (e.g., Yee and Murray 2004, O'Connor 2009), the rates of primary production (Raven and Geider 1988), or the plant traits that affect their susceptibility to herbivores (e.g., C:N ratio in Staehr and Wernberg (2009) or defensive secondary metabolites in Sudatti et al. (2011)).

In a natural way, seagrass meadows use to be exposed to several stressors simultaneously, that can act in a non-additive way, either synergistically or antagonistically (Darling and Côté 2008, Wernberg et al. 2012). Consequently, there is a pressing need to include multiple factors analysis in global change studies. Predicting the impacts of multiple stressors in organisms depend not only on measuring the impact of a single stressor on a single species but also to study the interactions among stressors, and how these affect the interactions between seagrass-consumer. Given the importance of herbivores in structuring marine communities (Poore et al. 2012), and the importance of primary producer nutritional quality in structuring seagrass food webs (Cebrián et al. 2009), changes in palatability of seagrasses promoted by climate change stressors may have the potential to alter seagrass communities.

Seagrass responses to herbivory damage

Seagrasses have developed a variety of mechanisms to avoid biomass loss by herbivores. The called "plant defense mechanisms" against herbivory may be categorised into three main strategies such as escape from consumers, resistance against herbivory or tolerance to be grazed. These mechanisms of defense are not mutually exclusive and include a range of structural, morphological and chemical adaptations, which can be regulated in response to consumers (Agrawal and Fishbein, 2006). In addition, we can find constitutive defenses, which function independently of the leaf damage and are always expressed in the plant, or

induced defenses that changes in the plants and are expressed when herbivore damage occurs (Karban and Baldwin, 1997). The induced defenses can involve both tolerance (i.e the ability of plants to withstand and survive to herbivory) and resistance traits (i.e leaf properties adaptations reducing leaf palatability) (Vergés et al. 2008). Few studies have addressed the tolerance and resistance responses to herbivory on seagrasses (e.g. Vergés et al. 2008, Sanmartí et al. 2014). These works have showed how *Cymodocea nodosa* and *Posidonia oceanica* were able to change a suite of plant traits to compensate biomass losses at different intensity of grazing (i.e. observing leaf growth, photosynthetic capacity, phenolic compounds, C:N ratios, etc.).

"The induced defenses are changes in plants that occur as a result of herbivore damage. They can involve both tolerance and resistance traits".

On the other hand, there are an increasing number of evidences showing that changes in the response to herbivory under climate change, can be crucial in determining seagrass community responses. For instance, Garthwin et al. (2014) showed that the tolerance of *Zostera muelleri* in simulated herbivory experiments to defoliation did not differ between warmed and unimpacted meadows, maintaining similar growth rates independently of temperature. Instead, Christianen et al. (2012) observed how grazing doubled leaf biomass production rates, while nutrients addition did not have an effect on these rates. In spite of these findings, few studies have considered the rate at which seagrasses replace damaged tissues or the processes that aid in this replacement, which has been shown to be important in other aquatic plant–herbivore interactions (Lehman and Scavia 1982, Bianchi 1988, Williams and Carpenter 1988, Sand-Jensen et al. 1994, Valentine and Heck 1999). Moreover, how these plants respond physiologically to this form of damage remains uncertain (see Valentine et al. 2004).

Methods employed in herbivory studies

There is an abundant scientific literature dealing with seagrass-dominated ecosystems with studies of herbivores and grazing effects on constituent primary producers, including both seagrasses and micro-, macro-algal epiphytes (Table 1). While some generalizations regarding plant-grazer interactions have been proposed, the diversity of experimental approaches used in studies on grazing relationships in seagrasses makes global comparisons difficult (Heck and Valentine 2006).

Table 1. Summary of selected studies or reports dealing with herbivory in seagrasses. Information is update from Valentine and Heck, 1999 and Valentine and Duffy, 2006 and the most relevant studies from the last 10 years.

Dugongs or manatees species	Seagrass species	Methodology	Reference
Trichehcus manatus	*Syringodium filiforme*	Field experiment based on percent cover and aboveground biomass assessment	(Provancha and Hall 1991)
Dugong dugon	*Halodule uninervis*	Observation and biomass monitoring	(De Longh et al. 1995)
Dugong dugon	*Zostera capricorni, Halophila ovalis, Halodule uninervis*	Field experiments based on monitoring biomass	(Preen 1995)
Dugong dugon	*Halophila ovalis, Halophila minor, Halodule uninervis, Halodule pinifolia, Thalassia hemprichii, Cymodocea rotundata, Cymodocea serrulata, Enhalus arcocoides, Syringodium isoetifolium*	Field observations during 7 years	(D'Souza et al. 2015)

Waterfowl species	Seagrass species	Study type	Reference
Anas acuta, Anas penelope, Anas plattyrhynchos, Branta bernicla	*Zostera noltei, Zostera marina*	Field experiment based on shoot density, biomass and percent cover were monitored	(Jacobs et al. 1981)
Anas crecca, Anas penelope, Branta bernicla	*Zostera noltei, Zostera marina*	Field experiment based on exclourses to monitor the percent cover recorded.	(Tubbs and Tubbs 1983)
Anas crecca, Anas penelope, Branta bernicla	*Zostera noltei, Zostera marina*	Field experiment based on exclosure experiment	(Madsen 1988)
Anas acuta, Authya americana, Anas platyrhynchos, Branta bernicla	*Zostera japonica, Zostera marina*	Field experiment based on bioenergetic study	(Baldwin and Lovron 1994)
Aythya americana	*Halodule wrightii*	Field monitoring of seagrass biomass during 2 years	(Mitchell et al. 1994)

Aythya americana	*Halodule wrightii*	Field monitoring of seagrass biomass	(Michot and Nault 1993)
Anas penelope, Branta bernicla	*Zostera sp.*	Field study based on changes in biomass with exclusion cages	(Portig et al. 1994)
Anas acuta, Anas penelope, Anas plattyrhynchos, Anas ferina, Fulicula atra, Netta rufina, Phoenicopterus ruber	*Ruppia maritima*	Field experiment based on separate exclosure designs excluded flamingos or all waterbirds to control plots	(Rodríguez-Pérez and Green 2006)
Cygnus atratus	*Zostera muelleri*	Field experiment monitoring of seagrass biomass and grazing marks	(Dos Santos et al. 2012)

Fish species	**Seagrass species**	**Study type**	**Reference**
Scarus spp., Sparisoma spp., Acanthurus spp.	*Thalassia testudinum, Halodule wrightii*	Field experiment based on stomach content	(Randall 1965)
Hyporhampus unifasciatus	*Ruppia maritima, Halodule wrightii*	Field experiment based on gut content	(Carr and Adams 1973)
Scarus guacamaia, Sparisoma radians	*Thalassia testudinum*	Fish bite marks identified	(Ogden and Zieman 1977)
Meuschemia freycinetti, Meuschenia trachylepsis, Monacanthus chinesis	*Posidonia australis*	Field experiment based on stomach content	(Bell et al. 1978)
Monacanthus chinesis	*Posidonia australis*	Field experiment based on stomach content and 14C labeled seagrass	(Conacher et al. 1979)
Sparisoma chrysopterum, Sparisoma rubripinne	*Thalassia testudinum*	Field tethering experiment	(Hay 1981)
Scarus croicensis, Sparisoma aurofrenatum, Acanthurus chiurugus, Acanthurus bahianus	*Syringodium filiforme, Thalassia testudinum*	Field tethering experiment	(Tribble 1981)
Scarid and siganid fishes	*Enhalus acoroides, Thalassia hemprichii, Halodule uninervis, Cymodocea rotundata, Syringodium isoetifolium*	Field based on monitoring bite marks	(Ogden and Ogden 1982)
Monacanthus ciliatus, Stephanolepsis hispidus	*Thalassia testudinum*	Field experiment based on stomach content	(Clements and Livingstone 1983)

Hyporhampus melanochir	*Zostera muelleri, heterozostera tasmanica*	Field experiment based on stomach content	(Roberston and Klumpp 1983)
Sparisoma chrysopterum, Sparisoma rubripinne	*Thalassia testudinum*	Field tethering experiment	(Lewis 1985)
Lagodon rhomboides	*Zostera marina*	Field experiment and laboratory bioenergetic and radioactive labeling study	(Montgomery and Targett 1992)
Arrhamphus sclerolepis krefftii	*No specific species of seagrass noted*	Histological examinations to determine ingested seagrass leaves	(Tibbetts 1997)
Sparisoma radians	*Thalassia testudinum*	Field tethering experiment	(Kirsch et al. 2002)
Sparisoma radians	*Thalassia testudinum*	Feeding preference assays	(Goecker et al. 2005)
Scarid fishes	*Thalassia hemprichii, Enhalus acoroides, Cymodocea rotundata, Halophila ovalis*	Field based on monitoring bite marks and tethering experiment	(Unsworth et al. 2007)
Sarpa salpa	*Posidonia oceanica*	Field fertilization experiment based on changes in fish feeding behavior	(Prado et al. 2010)
Sarpa salpa	*Posidonia oceanica*	Paired feeding assays	(Vergés et al. 2011)
Sparisoma cretense	*Cymodocea nodosa*	Field observations and laboratory assays	(Del Río et al. 2016)

Turtle species	**Seagrass species**	**Study type**	**Reference**
Chelonia mydas	*Thalassia Testudinum*	Field experiment based on bioenergetic and observation study	(Bjorndal 1980)
Chelonia mydas	*Thalassia hemprichii, Cymodocea rotundata*	Field experiment using exclosures	(Kelkar et al. 2013)

Sea urchin species	**Seagrass species**	**Study type**	**Reference**
Lytechinus variegatus	*Thalassia testudinum*	Field observations	(Camp et al. 1973)
Lytechinus variegatus	*Thalassia testudinum*	Field observations	(Bach 1979)

Tripneustes gratilla	*Halophila stipulacea*	Field observations	(Lipkin 1979)
Astopyga magnifica	*Zostera marina*	Field experiment based on gut content	(Bak and Nojima 1980)
Paracentrotus lividus	*Posidonia oceanica*	Field experiment based on grazing impact in seagrass biomass and density	(Kirkman and Young 1981)
Lytechinus variegatus	*Thalassia testudinum*	Field observations and gut content. Feeding preference assays	(Vadas et al. 1982)
Lytechinus variegatus, Tripneustes ventricosus	*Thalassia testudinum*	Field experiment using exclosures	(Keller 1983)
Heliocidaris erythrogramma	*Posidonia australis*	Field observations	(Larkum and West 1990)
Lytechinus variegatus	*Thalassia testudinum*	Feeding preference assays	(Montague et al. 1991)
Lytechinus variegatus	*Thalassia testudinum*	Field experiment using exclosures	(Valentine and Heck 1991)
Tripneustes gratilla, Salmacis phaeroides	*Thalassia hemprichii*	Field and laboratory measurments of sea urchin consumption. Feeding preference assays	(Klumpp et al. 1993)
Lytechinus variegatus	*Thalassia testudinum*	Field experiment using exclosures	(Heck and Valentine 1995)
Lytechinus variegatus	*Thalassia testudinum*	Two field experiment variying the duration of sea urchin grazing and examining the effects of low levels of grazing onseagrass growth and biomass	(Valentine et al. 2000)
Tripneustes gratilla	*Thalassodendron ciliatum*	Field experiments based on seagrass density changes combining with mathematical estimates	(Alcoverro and Mariani 2002)
Paracentrotus lividus	*Posidonia oceanica*	Field experiments based on stomach content in seagrass near and away CO_2 vents	(Nogueira et al. 2017)

Crustacean species	Seagrass species	Study type	Reference
Ampithoe spp.	*Syringodium isoetifolium*	Laboratory experiment based on ingestion rates	(Mukai and Lijima 1995)
Gammarus mucronatus, Cymadusa compta, Ampithoe longimana	*Zostera marina*	Laboratory experiments	(Duffy and Harvilicz 2001)
Gammarus locusta	*Zostera marina*	Laboratory experiments	(Andersson et al. 2009)

Gastropod species	Seagrass species	Study type	Reference
Tectura depicata	*Zostera marina*	Laboratory experiment based on seagrass grazed physiology	(Zimmerman et al. 1996)
Thalotia conica	*Posidonia sinuosa*	Laboratory assays	(Jernakoff and Nielsen 1997)
Smaragdia viridis	*Zostera marina*	Field and laboratory experiment based on feeding activity	(Rueda et al. 2009)
Bittium reticulatum, Gibbula ardens, Jujubinus exasperatus, Tricolia pullus, Alvania mantagui, Jujubinus striatus	*Posidonia oceanica*	Laboratory experiment using biofilms on artificial substrata	(Castejón-Silvo and Terrados 2017)

Mixing grazers species	Seagrass species	Study type	Reference
Waterfowls: *Anas acuta, Anas carolinensis, Branta canadensis.* **Fish:** *Microcottus sellaris.* **Gastropod:** *Margarites helicinus, Littorina sitkana, Lacuna variegata.* **Crustacean:** *Telmessus chierogonus*	*Zostera marina*	^{13}C:^{12}C analysis	(McConnaughey and McRoy 1979)

Waterfowls: *Anas acuta, Anas crecca, Anas penelope, Anas platyrhynchos, Authya ferina, Branta bernicla, Cygnus olor, Fulica atra.* **Crustacean:** *Idotea chelipes*	*Zostera marina*	Field experiment based on bioenergetic study and laboratory experiment	(Nienhuis and Groenendijk 1986)
Sea urchin: *Diadema antillarum.* **Turtle:** *Chelonia mydas*	*Thalassia testudinum*	Field experiment based on changes in seagrass growth nad biomass	(Zieman et al. 1984)
Fish: *Hyporhampus melanochir.* **Crustacean***: Nectocarcinus inegrifons*	*Posidonia australis*	Gut content experiment	(Nichols et al. 1986)
Fish: *Lagodon rhomboides, Stephanolepis hispidus, Nicholstina usta.* **Sea urchin:** *Lytechinus variegatus*	*Thalassia testudinum, Halodule wrightii, Syringodium filiforme*	Feeding preference assays	(Prado and Heck 2011)
Fish: *Sarpa sapla.* **Sea urchin:** *Paracentrotus lividus.*	*Posidonia oceanica*	Quantifiy of leaf tips frequency	(Ruiz et al. 2001)
Fish: *Sarpa sapla.* **Sea urchin:** *Paracentrotus lividus.*	*Posidonia oceanica*	Tethering experiment and bite marks	(Tomas et al. 2005a)
Fish: *Sarpa sapla.* **Sea urchin:** *Paracentrotus lividus.*	*Posidonia oceanica*	Field experiments using exclosures	(Tomas et al. 2005b)
Fish: *Sarpa sapla.* **Sea urchin:** *Paracentrotus lividus.*	*Posidonia oceanica*	Tethering experiment and bite marks	(Prado et al. 2007)
Fish: *Sarpa sapla.* **Sea urchin:** *Paracentrotus lividus.*	*Posidonia oceanica*	Field experiment based on bite marks	(Pinna et al. 2009)
Dugonds, turtles, fishes and mesograzers. Non identified	*Zostera muelleri*	Field experiments using exclosures	(Ebrahim et al. 2014)
Fish: *Sarpa sapla.* **Sea urchin:** *Paracentrotus lividus.*	*Posidonia oceanica, Cymodocea nodosa*	Tethering experiment and laboratory assays	(Marco-Méndez et al. 2015)

a) Indirect vs direct methods

In early studies, indirect approaches were mainly used by ecologists following the methodology applied in terrestrial studies, using methods such as counting grazing marks on leaves, comparing leaf density or biomass at grazed and ungrazed sites, using traditional exclusion cages (e.g. Poore et al. 2012) or the recent method of bubble curtains (Bennet et al. 2017) and laboratory ingestion studies as well. These indirect methods to estimate grazing on seagrasses have yielded low rates of herbivory and have been based on static measures of leaf loss to grazers made once or a few times annually (Cebrián, et al. 1996a, Cebrián et al. 1996b)

Currently, a re-assessment of herbivory rates estimations have been done through direct methods such as tethering experiments. These studies have demonstrated that herbivory can be a key process in seagrass ecosystems (Kirsch et al. 2002, Tomas et al. 2005a, Prado et al. 2007). It is an accurate method to quantify consumption in a short time period. However, tethers can influence feeding behaviour and consumption rates and also can alter plants status as a result of leaf manipulations. Direct methods showed herbivory can be determine the structure and distribution of temperate seagrass ecosystems (Tomas et al. 2005a) and can be highly variable spatial and temporally (Prado et al. 2007). Wide discrepancies encountered when comparing direct and indirect methods suggest achieving accurate estimates of herbivory processes. Then, further studies are required to assess the real importance of herbivory on seagrass ecosystems and its ecological implications.

b) Food choice experiments

Herbivory studies often employed food choice experiments with different diet combinations to test how food ingestion, previously observed in the field, could be related to food selectivity or preference by herbivores (Vergés et al. 2007b, 2011, Prado and Heck 2011). This methodology allows to isolate other factors (e.g. from other food sources or predators), that may interfere with feeding behaviour in the field, and also enables testing how factors related to leaf traits can influence herbivore decisions (Tomas et al. 2011a). Negatively, working with wet weights can be less accurate and paired replicates can influence feeding behaviour and consumption rates. Anyway, food choice experiments have demonstrated that leaf properties related with food quality such as nutritional, structural, chemical or biomechanical traits can influence feeding decisions differently, depending on herbivore species (Prado and Heck 2011). Hence, this kind of experiments may also help us to understand consumption patterns recorded by tethering experiments, or previous feeding behaviour observed in the field under natural conditions.

c) Further methods

Among methods for quantifying dietary contribution, stomach content analysis is the most accurate, although it gives food information relative to a short time period and requires sacrifice of the animal (Legagneux et al. 2007). Feeding observations allow monitoring in natural conditions without experimental interference. However, observation should be highly replicated in space and time especially in the case of mobile species. Stable isotope analysis is other method that can provide non-destructive and time –integrative information about the diet and origin of food sources (Inger and Bearhop 2008; Piñón-Gimate et al. 2016). Under appropriate conditions, it is possible to use the stable isotope ratios in tissues to quantify the relative importance of different dietary items to a consumer, based on the premise that consumer's tissues will resemble the long-term isotopic composition of the diet (Fry and Sherr 1984).

Objectives

The main hypothesis of this PhD Thesis is to analyze how environmental conditions, such as hydrodynamics, nutrients and global change (i.e. warming and acidification) modulate the seagrass-grazers interaction through two-ways: (1) how hydrodynamics, eutrophication and global change factors (i.e. warming and acidification) influence on seagrass palatability through the variation of leaf traits and (2) how the interaction between hydrodynamics and seagrass canopy modulate the feeding capacity of grazers. In addition to this main objective, this PhD Thesis analyzes the response to biomass loss by herbivory according to colonization states of seagrass patches after a perturbation event.

This main goal was accomplished combining *in situ* manipulative and mesocosm experiments, where the changes in leaf traits (i.e. morphological, nutritional, structural, chemical and biomechanical traits) were analysed and the resulting feeding rates by generalist herbivores were assessed.

The aforementioned main objective was reached through four specific objectives:

1. To test how the interaction between hydrodynamics and leaf traits modulates seagrass palatability and feeding capacity of grazers (**Chapters 1** and **chapter 2**).

2. To examine how intra-specific differences in the two seagrass species *Cymodocea nodosa* and *Zostera noltei* determine the plant palatability and consumption rates by herbivores constrained by diversity and abundance of food. **(Chapter 3)**

3. To evaluate the effects and interactions among eutrophication and the main factors of global change (i.e. warming and acidification) on leaf traits of *Cymodocea nodosa* and implications on herbivory process. (**Chapters 4 and chapter 5**)

4. To explore the consequences of loss biomass and mechanical damage on leaves produced by herbivores in the tropical seagrass *Halodule wrightii* with different colonization states (**Chapter 6**).

Outline of the Thesis

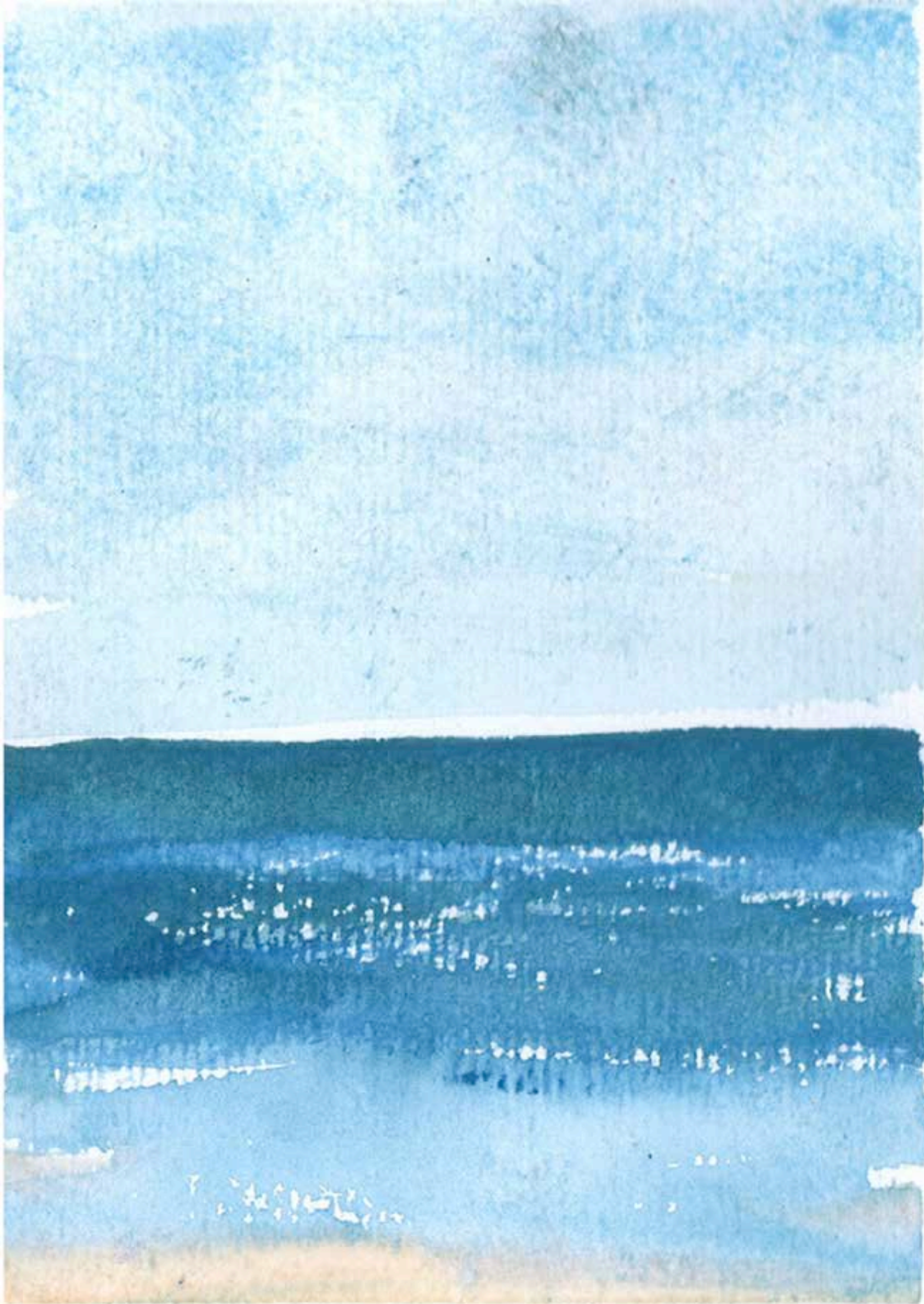
The ability to understand how changes in environmental factors, such as hydrodynamic conditions or nutrients availability and those resulting from global change (i.e. warming and acidification) alter plant characteristics (in terms of palatability), and thus influence the feeding capacity of grazers, will improve our knowledge on the functioning and structure of seagrass communities. Therefore, the main goal of this PhD Thesis was to examine a suit of plant features (nutritional, biomechanical, morphological, structural and chemical), which may be modified by environmental conditions and global change factors, in order to untangle the feeding capacity of seagrass grazers and the complex interrelationships between different environmental conditions (natural and human-induced) and seagrass herbivory. Moreover, seagrass induced responses to loss biomass due to herbivory at physiological level were addressed under different colonization statess of natural recovered patches.

The first part of the PhD Thesis involves three studies on the acclimation of temperate seagrasses to different environmental conditions, under controlled and field conditions. In **chapter 1**, a controlled hydrodynamic experiment using a large race-track flume was performed to untangle the interactive effect of seagrass canopy complexity and unidirectional flow velocity, on the capacity of mesograzers to keep seagrass leaves clean by feeding on epiphytes. In **chapter 2**, sheltered and unsheltered *Cymodocea nodosa* meadows were compared in four areas under annual cross-transplantation experiment. In addition, in each sample site were transplanted shoots from the different location in order to demonstrate the influence of leaf palatability. Direct measurements of grazing were evaluated to elucidate how different hydrodynamics affect leaf defoliation. In the **chapter 3**, different marine macrophytes, including two species of seagrass such as *C. nodosa* and *Zostera noltei*, which inhabits several locations with different degrees of exposure to currents, were characterised in relation to their morphological, nutritional and structural traits. Then, they were offered to *Paracentrotus lividus* studying the relationships among leaf properties. Differences in leaf palatability were analysed in an attempt to understand how consumption rates over seagrasses are modified by their intra and inter-specific differences and with the presence of other macrophytes with different palatability that co-occur in seagrass communities.

The second part of this Thesis comprises two chapters on the acclimation of the temperate seagrass *Cymodocea nodosa* to global change factors (eutrophication, acidification and warming) under controlled and field condition and their influence on seagrass palatability. The **chapter 4** included a manipulative field experiment to study the eutrophication effects on leaf

traits in *C. nodosa* meadows. The influence of the epiphyte presence in leaves was also tested. The correlations of a suite of leaf traits underlying food preferences by seagrass consumers (nutritional, structural and biomechanical traits) were analysed, validating the hypothesis that species inhabiting in eutrophicated areas may be potentially more attractive to herbivores. In **chapter 5**, the acclimation process of *C. nodosa* was studied under the effects of three main factors of global change: temperature, acidification and nutrients input. The study aimed to elucidate if the combination of these factors had an interactive effect on leaf traits and whether such changes determined food selection by *P. lividus*.

The last part focused on the seagrass induced responses against biomass loss and mechanical damage promoted by herbivory in natural meadows at different colonization states. In **chapter 6** simulated herbivory was performed in three patches of the tropical seagrass *Halodule wrightii*, after a natural perturbation event. Changes in biomass, leaf growth, carbon and nitrogen content in tissues and total non-structural carbohydrates were evaluated from three patches with contrasting colonization states to test the existence of stage-dependence in responses to simulated grazing.



CHAPTER 1

The interactive role of hydrodynamic flow and shoot density as a top-down regulator in seagrass meadows

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Aprender nunca dejará exhausta la mente.
Learning will never leave the mind exhausted.

Leonardo da Vinci

The role of hydrodynamic flow combined with habitat complexity as a top-down regulator in seagrass meadows

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Abstract

In this study, we addressed the combined effects of hydrodynamic regime and seagrass shoot density on the feeding and foraging behaviours of mesograzers. A full factorial experiment, with flow velocity (high, medium and low) and shoot density (high vs. low) as main factors, was conducted in a racetrack flume using artificial seagrass beds. The results show that when high flow velocity conditions were combined with low shoot density, mesograzers consumption of epiphytes was strongly reduced. In contrast, when hydrodynamic forcing was low or shoot density was high, mesograzers exhibited high feeding rates and vigorous swimming behaviour. These results clearly indicate that high hydrodynamic stress reduces the time that mesograzers can spend feeding and inhibits their swimming behaviour, thus indirectly impacting the density of epiphytes. The triggering of trophic cascades in seagrass communities under these experimental conditions depended on the interrelationship and feedbacks among shoot density, abiotic (flow velocity) and biotic (epiphytes and mesograzers) compartments, with flow velocity exerting a top-down control on seagrass ecosystems.

Introduction

Over the last decades, a central focus in ecological research has been deciphering how environmental forcing and biological interactions are involved in the transfer of primary production to higher trophic levels (Duffy and Hay 2000, Duffy and Harvilicz 2001). Understanding the interplay between bottom-up and top-down processes is critical given the ongoing perturbations of environmental conditions and subsequent effects on food-web topology (Duffy 2003; Duffy, Richardson and France 2005; Byrnes, Reynolds and Stachowicz 2007; Poore et al. 2012). Seagrass ecosystems serve well as model communities to study the importance of bottom-up and top-down processes (Duffy et al. 2005; Heck and Valentine 2007; Burkholder et al. 2013) because they are highly threatened by both eutrophication (i.e. bottom-up) and overfishing (top-down) processes, besides other human activities (Waycott et al. 2009). In addition, seagrass ecosystems are hotspots for biological diversity since they are considered to be foundation species and, thus, can ameliorate biotic and/or abiotic stress, which facilitates the development of other species (Orth et al. 2006; van der Heide et al. 2012).

Earlier studies have revealed that a large number of seagrass-associated organisms feed heavily on epiphytic algae, which are important primary producers in seagrass ecosystems and make a significant contribution to food webs (see Borowitzka and Lavery 2005 and references therein). Overproduction by epiphytic algae, however, has been associated with large-scale losses of seagrasses in eutrophic waters (Silberstein, Chiffings and McComb 1986; Walker and McComb 1992). Grazers that feed on epiphytes can exert significant top-down control, preventing the negative consequences of epiphyte overgrowth (Jernakoff et al. 1996; Duffy et al. 2005; Borowitzka et al. 2006; Heck and Valentine 2006; Valentine and Duffy 2006; Poore et al. 2012). The regulation of the epiphytic community by grazers can initiate a trophic cascade, indirectly affecting seagrasses in eutrophicated areas (Williams and Ruckelshaus 1993; Philippart 1995; Schanz, Polte and Asmus 2002; Moksnes et al. 2008), since grazing improves the light environment and, thus, growth conditions while boosting the transfer of matter and energy to higher trophic levels (van Montfrans et al. 1984; Orth 1992; Jernakoff and Nielsen 1997; Valentine et al. 1997; Garcia, Littler and Littler 1999; Lavery et al. 2007). The important role of grazers on seagrass ecosystems raises the question of which factors control mesograzer activity in such communities.

Most studies on the regulation of epiphyte grazers have focused on how predation and habitat complexity influence their abundance and distribution patterns (Orth 1992; Duffy and Harvilicz 2001; Edgar and Klumpp 2003; Duffy *et al.* 2007; Cook, Vanderklift and Poore 2011). In contrast, the effects of physical factors like flow velocity on grazing activity have been rarely examined (but see Schanz et al. 2002, Lavery et al. 2007) even though

hydrodynamics may be expected greatly influence both abiotic and biotic compartments. For instance, hydrodynamics are known to affect light conditions (Drake, Dobbs and Zimmerman 2003), nutrients and resource transfer within the canopy (Cornelisen and Thomas 2004, Morris et al. 2008, González-Ortiz et al. 2014b), which in turn can affect epiphytes (Borowitzka *et al.* 2006; Lavery *et al.* 2007), seagrasses (Peralta *et al.* 2006; De Los Santos *et al.* 2009) and fauna in positive or negative ways (Grizzle et al. 1992, Irlandi 1996, Schanz et al. 2002, González-Ortiz et al. 2014b). Therefore, hydrodynamics may be expected to affect bottom-up and top-down control processes in complex ways. The strength of the interactions involved may depend on the magnitude of both biotic and abiotic variables, such as the complexity of the seagrass canopy (i.e. shoot density) and physical forcing. In this study, we aim to untangle the interactive effects of seagrass canopy complexity and current velocity on the capacity of mesograzers to keep seagrass leaves clean by feeding on epiphytes, under controlled hydrodynamic conditions (i.e. using a large race-track flume that generates a unidirectional flow). In doing so, we also aim to gain insight into the underlying mechanisms behind these processes.

Materials and methods

Artificial canopy design

Seagrass mimics were designed to simulate the main physical properties of these macrophytes when underwater. We used two different designs for mimics depending on their function in the experiments:

1) *Bare plastic mimics* were used to create an artificial seagrass meadow without epiphytes. These mimics were used to form matrices of contrasting complexity, in which a limited number of *epiphytized mimics* were subsequently placed. Shoots of the *bare plastic mimics* were simulated by using a group of leaf-like plastic straps, which were attached to a wooden stick simulating the rhizome-root system using a 4 x 0.4 cm plastic straw filled with adhesive silicon (imitating the leaf sheath) (González-Ortiz et al. 2014a and 2016). Morphometric characteristics of the mimicked leaves (length, width and thickness; see Figure1) resembled those of the main species thriving in European Atlantic coasts: *Zostera noltei*, *Z. marina* and *Cymodocea nodosa* (González-Ortiz et al. 2014a). Different treatments involved manipulating the architectural characteristics of the bed (e.g. shoot density), which was facilitated by the use of mimics (Bouma et al. 2005, Peralta et al. 2008, González-Ortiz et al. 2014a).

2) *Epiphytized mimics* were used to avoid any kind of potential biological interactions between the seagrass and animals (e.g., grazing, herbivory or chemical interactions), thus allowing the study to be constrained by the interactions between epiphytes and mesograzers.

These *epiphytized mimics* were constructed using a silicon tube sealed at both ends to resemble shoots, which was attached to a wooden stick and anchored to the sediment (Brun et al. 2012, González-Ortiz, et al. 2014b; see Figure1). The silicon tube floated underwater and bowed with the flow, since the central air chamber provided positive buoyancy. In order to induce epiphyte colonization of the mimics, they were kept in outdoor tanks filled with natural seawater renewed by tidal action for a month under natural light and temperature conditions. Care was taken to obtain similar epiphyte cover between mimics to enable homogenized experimental conditions. This procedure promotes the development of a dense epiphytic film cover over these mimics, as already observed in previous studies (González-Ortiz et al. 2014a).

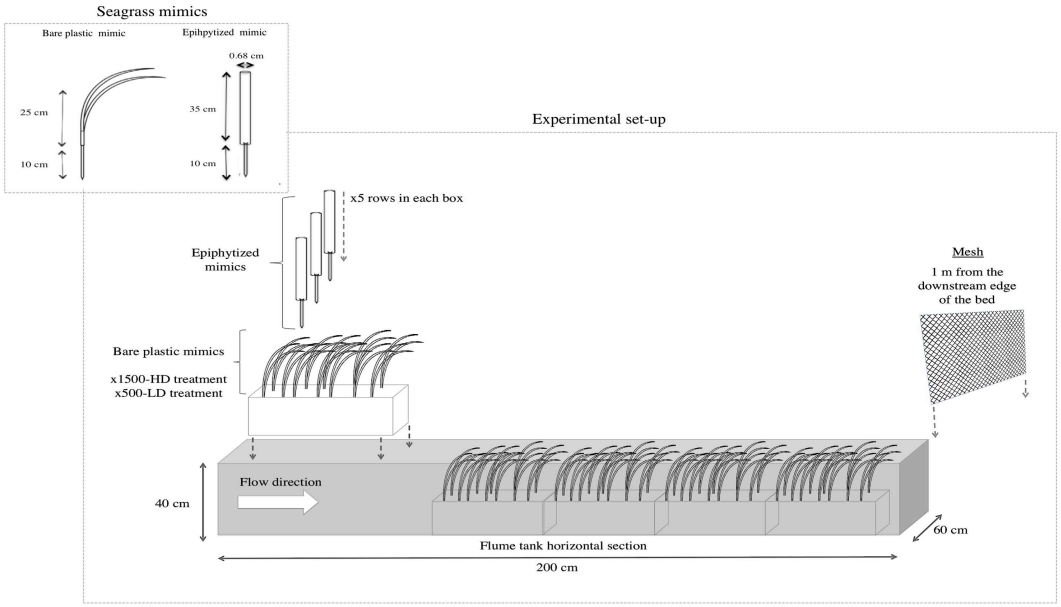

Figure 1. Drawing of the experimental set-up and the two different designs for seagrass mimics used in the feeding experiments.

Grazer selection

We used the amphipod *Gammarus locusta* as a model grazer in this study. *G. locusta* is a mesograzer that occurs naturally in seagrass beds on North Atlantic coasts (Baden and Pihl 1984; Fredriksen et al. 2005; Baden et al. 2010). Individuals were collected from the Oosterschelde Estuary (The Netherlands). Tidal currents for such estuarine marshes ranged from 5 to 40 cm $\cdot s^{-1}$ (Bouma et al. 2005). Natural density of mesograzers was measured in the field using a 400 cm^2 quadrat at 10 different selected ramdomly areas into the *Zostera* beds. Mesograzers natural density was 15 ± 6.2 ind $\cdot m^2$. The experimental amphipods were collected using "*Ulva* traps". These traps comprising a series of mesh bags containing fresh *Ulva* sp.,

which is the major component of the natural *G. locusta* diet (Costa and Costa 2000), were placed at low tide and collected after a tidal cycle. A mesh size of 10 mm was used to collect only sexually mature *G. locusta* individuals (> 7 mm of length; Andersson et al. 2009). The length of the amphipods was measured as the distance between the anterior end of the rostrum and the posterior end of the last urosome, which is represented by the last three abdominal somites bearing modified appendages in amphipods (see Moore and McCormick 1969), when the amphipod is in an extended position, i.e. pressed flat against a surface. *G. locusta* length was on average of 10.4 ± 1.2 mm (n = 180). The amphipods were kept outdoors in 60 L flow-through tanks (100–200 individuals per tank) for at least 5 days before the experiment, with *Ulva* sp. provided as both substratum and food source for each tank.

Pilot experiment

A series of pilot trials were conducted to ascertain that the feeding behaviour of *G. locusta* remained similar when offered mimics with epiphytes vs. natural seagrass shoots with epiphytes. These treatments were designed to account for the effect of using mimics rather than natural seagrass shoots. *Epiphytized mimics* were selected from the pool of mimics produced in the outdoor tanks (see above). Natural shoots of *Z. marina* were collected from *Zostera* meadows on the mudflats of Viane (51º39'N, 4º01'E), the Oosterschelde, in the southwestern part of the Netherlands. After collection, they were cleaned of epiphytes and planted in the same outdoor reservoirs as explained above. Here, they were left to be colonized by epiphytes under the same conditions as described for the *epiphytized mimics*.

We tested the following treatments in the pilot experiment: (1) high density of *epiphytized mimics* (HD-EM, n=10 mimics), (2) high density of naturally epiphytized *Z. marina* shoots (HD-EZ, n= 10 shoots), (3) low density of *epiphytized mimics* (LD-EM, n= 5 mimics) and (4) low density of naturally epiphytized *Z. marina* shoots (LD-EZ, n= 5 shoots). To estimate the initial epiphyte content, a total of 10 *epiphytized mimics* and 10 *Zostera marina* shoots were scraped and epiphyte biomass was quantified. Each treatment was carried out in triplicate and was conducted in tanks (50 x 35 x 30 cm) filled with natural seawater and sand. To minimize disturbance, no aquarium air pumps were used, and all aquaria were surrounded by opaque plastic. Before each trial, individuals of *G. locusta* were randomly selected from the reservoir tanks and kept without food for 24 h (maximum starvation time) to remove chlorophyll from the stomach content (i.e. starved grazers). At the beginning of each trial, five grazers were placed in each tank and after 6 h, they were removed from the water and immediately frozen at -20ºC for further analysis (i.e. experimental grazers). In addition, another five *G. locusta* from the starved pool were also frozen before each run to estimate the initial chlorophyll content of the stomach (Ritchie 2006). Feeding rate was calculated as the

difference in chlorophyll a (Chla) content between experimental grazers and starved grazers, taking into account the experimental period.

Racetrack flume set-up

The experiment was run in a large unidirectional racetrack flume tank (Bouma et al. 2005) with a testing area of 200 x 60 cm and a total length of 1,700 cm. The flume tank was filled with natural seawater (water column height of 0.4 m). Five wooden boxes (40 x 60 x 10 cm) were filled with natural sand and planted with *plastic mimics* (see González-Ortiz et al. 2014b), creating artificial beds with two different densities (i.e. representing 2 different levels of habitat complexity): (1) high density (HD, 1,500 mimics $\cdot m^{-2}$) and (2) low density (LD, 500 mimics $\cdot m^{-2}$). For each run, a total of 75 *epiphytized mimics* from the initial pool were placed in 3 rows every 25 cm within the artificial bed formed by 5 boxes, with a single density each one, along the flume tank, so that all experimental space had the same epiphyte load. Consequently, *G. locusta* were subjected to the same probability of finding food (i.e. *epiphytized mimics*) across the artificial canopy. At the beginning of each run, 10 *epiphytized mimics* from the initial pool of mimics were scraped to measure the initial epiphyte load for each trial. At the end of the channel (1 meter far from the downstream edge of the bed) a net (mesh size of 5 mm) was placed to prevent the escape of grazers further downstream along the flume that could return to the leading edge of the reconstructed bed because of the circular shape of the flume (Figure 1).

Racetrack flume experiment

A total of 18 tests were performed for two different canopy densities (HD and LD) under low (0.05 m $\cdot s^{-1}$), medium (0.15 m $\cdot s^{-1}$) and high (0.30 m $\cdot s^{-1}$) unidirectional flow velocities (LV, MV and HV, respectively). The flume was run at the given speed for at least 15 min before starting the tests to stabilize the hydrodynamic regime. Within the flume tank, the X-, Y- and Z-axes are oriented respectively to the main flow direction (X), perpendicular to the lateral flume tank walls (Y), and vertically (Z) (Peralta et al. 2008, González-Ortiz et al. 2014b). After this stabilization period, 10 starved individuals of *G. locusta* were haphazardly introduced within the artificial seagrass bed. In addition, 10 starved *G. locusta* were frozen before each run to estimate the initial chlorophyll stomach content. The racetrack flume was left running for 6 hours each trial under dark conditions to minimize disturbance of the amphipods.

During the experimental period, the positions of *G. locusta* inside the artificial bed were checked four times along the transparent wall of the test section: observation 1 at 10 min, observation 2 at 1 h, observation 3 at 3 h, and observation 4 at 6h, the end of the experiment. This data was collected manually by observing the organisms along the X, Y and

Z axes of the flume tank, using a dim flashlight since the amphipods should remain in the dark as much as possible. We noted the number of individuals actively swimming during the observations. Once the experimental period ended, grazers were removed and immediately frozen at -20ºC for Chla analysis (Ritchie, 2008). Feeding rate was calculated as the difference in Chla content between grazers at the end of the experiment and initial starved grazers over the experimental period. Experimental *epiphytized mimics* were collected and scraped for epiphytes, which were then dried and weighed. Moreover, the same experimental set-up was run for all aforementioned treatments (n = 18), but without including *G. locusta* (no-grazer controls), in order to determine the effects of hydrodynamics in epiphyte distribution.

Statistical analyses

Significant differences in Chla content in the pilot and racetrack flume experiments were checked using a 2-way fixed factor ANOVA (2 levels of shoot density and 3 hydrodynamic regimes). Data normality and homoscedasticity were checked before applying ANOVA. To test for significant differences in epiphyte load in each run, a non-parametric Kruskal-Wallis test was used since data were not normally distributed even after applying several different transformations (i.e. epiphyte load in high density tests for grazer and no-grazer treatments, and *G. locusta* position along the racetrack flume). Data are presented as mean ± s.e. (standard error) and significance levels were set at p = 0.05. Statistical analyses were computed with R statistical software 3.0.2 (R Development Core Team 2013).

Results

Pilot experiment

There were no differences in epiphyte load between natural *Zostera* shoots and *epiphytized mimics* from the initial pool (1.65 ± 0.27 *vs* 1.75 ± 0.13 g DW shoot^{-1} or mimic^{-1}, respectively; p-value = 0.06; n = 10). Our food preference experiment demonstrated that *G. locusta* had the same feeding rate whether exposed to epiphytes on *epiphytized mimics* or natural *Zostera* shoots (p-value = 0.255), independent of food availability (i.e. high or low food availability). Also, 6 h was shown to be sufficient to detect changes in Chla content in *G. locusta* (Figure 2 and Table 1).

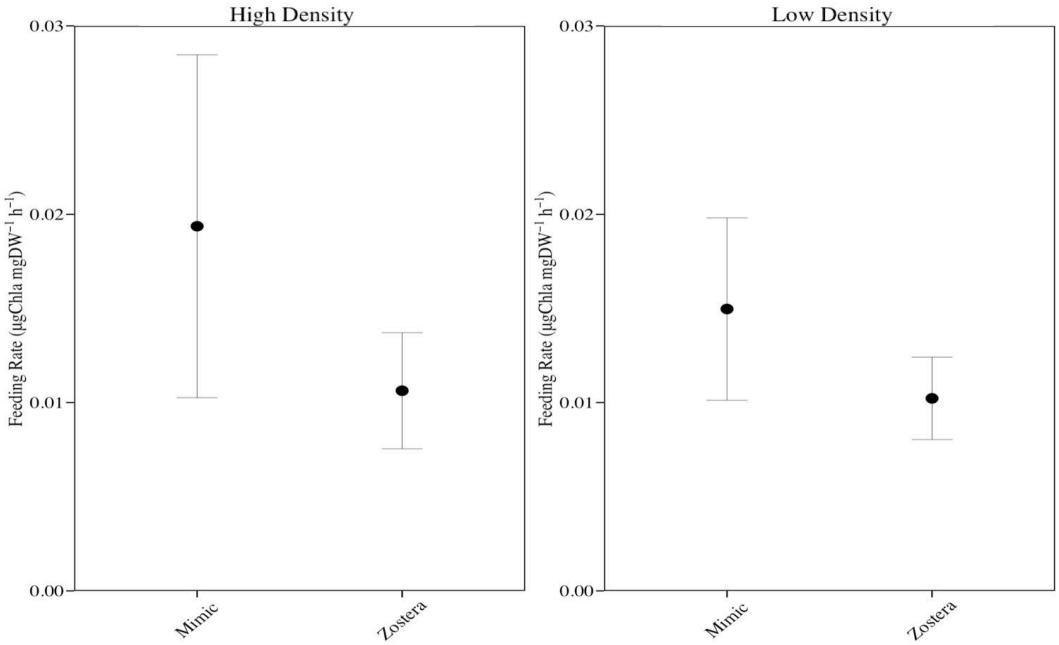

Figure 2. *Gammarus locusta* feeding rate (n=3) in the pilot trials at different levels of shoot density in *epiphytized mimic* and *Zostera* canopies (n= 12) Values were normalized by epiphyte load in each tank (g DW epiphytes). Bars indicate standard errors.

Table 1. Statistical results of the 2-way ANOVA analysis for the pilot trials examining the effects of food type (mimics or *Zostera* canopy) and shoots density on *G. locusta* feeding rate. Significance level was set at p-value < 0.05.

	df	MS	F	p
Food Type	1	1.362×10^{-4}	1.506	0.255
Density	1	1.730×10^{-5}	0.191	0.673
Food Type-Density	1	1.193×10^{-5}	0.132	0.726
Residuals	8	9.044×10^{-5}		

Racetrack flume experiment: feeding behaviour of Gammarus locusta

Flow velocity and shoot density (i.e. density of mimics) significantly influenced the feeding rate of *G. locusta* (p-value < 0.01 and p-value = 0.018 respectively, Table 2*)*. In contrast, the interaction between flow velocity and density (p = 0.174) was not significant. In all the HV treatments, independent of density, Chla content in *G. locusta* after 6 h was so low that it was not detectable with the method used, yielding a feeding rate of zero (Figure3). However, a remarkable increase of Chla in *G. locusta* was recorded under MV and in LV treatments, especially under HD conditions. Although shoot density had no influence during HV treatments, during MV and LV treatments, we found significantly higher feeding rates under high shoot density (both MV and LV, p-value < 0.01) compared with low shoot density (Figure3).

Table 2. Statistical results of the 2-way ANOVA analysis examining the effects of flow velocity and shoot density on *G. locusta* feeding rate. Bold font indicates significant differences (p-value < 0.05).

	df	MS	F	p
Flow velocity	2	0.034	20.16	**0.00014**
Density	1	0.012	7.41	**0.018**
Flow velocity-Density	2	0.003	2.027	0.174
Residuals	12	0.002		

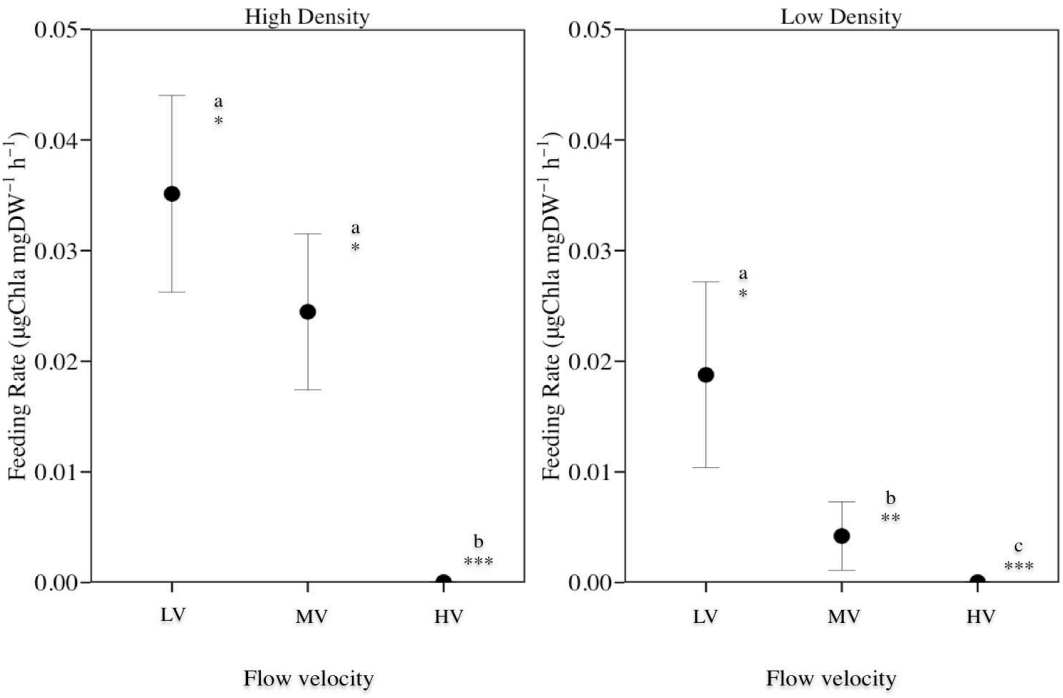

Figure 3. *G. locusta* feeding rate (n= 3) in the racetrack flume tank experiments for different unidirectional flow velocities (low (LV) = 0.015, medium (MV) = 0.045 and high (HV) = 0.30 m s^{-1}) and shoot density (high and low density) (n= 18). Letters indicate significant differences between flow velocities within each level of density. Asterisks indicate significant differences between density levels at the same velocity treatment (p-value < 0.05).

Change in epiphyte load along artificial bed

Epiphyte load showed a spatially explicit distribution depending on flow velocity and shoot density (Table 3). Overall, a significant decrease was found at the leading edge of the artificial bed (i.e. box 1, p-value < 0.01), especially under HV treatments (Figure4). Both flow velocity and flume position affected epiphyte load under LD treatments. However, hydrodynamic regime had no influence on epiphyte load under HD treatments (Table 3, Figure 4). Similar results were found in the no-grazer treatments, since an overall reduction (lower than 6 ± 11.08%) in epiphyte load was found in the leading edge (box 1) of all the treatments, which was more noticeable under high velocity and low shoot density (Table 3). In addition, hydrodynamics had not effects on epiphyte load under HD treatments (p-value = 0.93) as already found when *G. locusta* was present (Table 3).

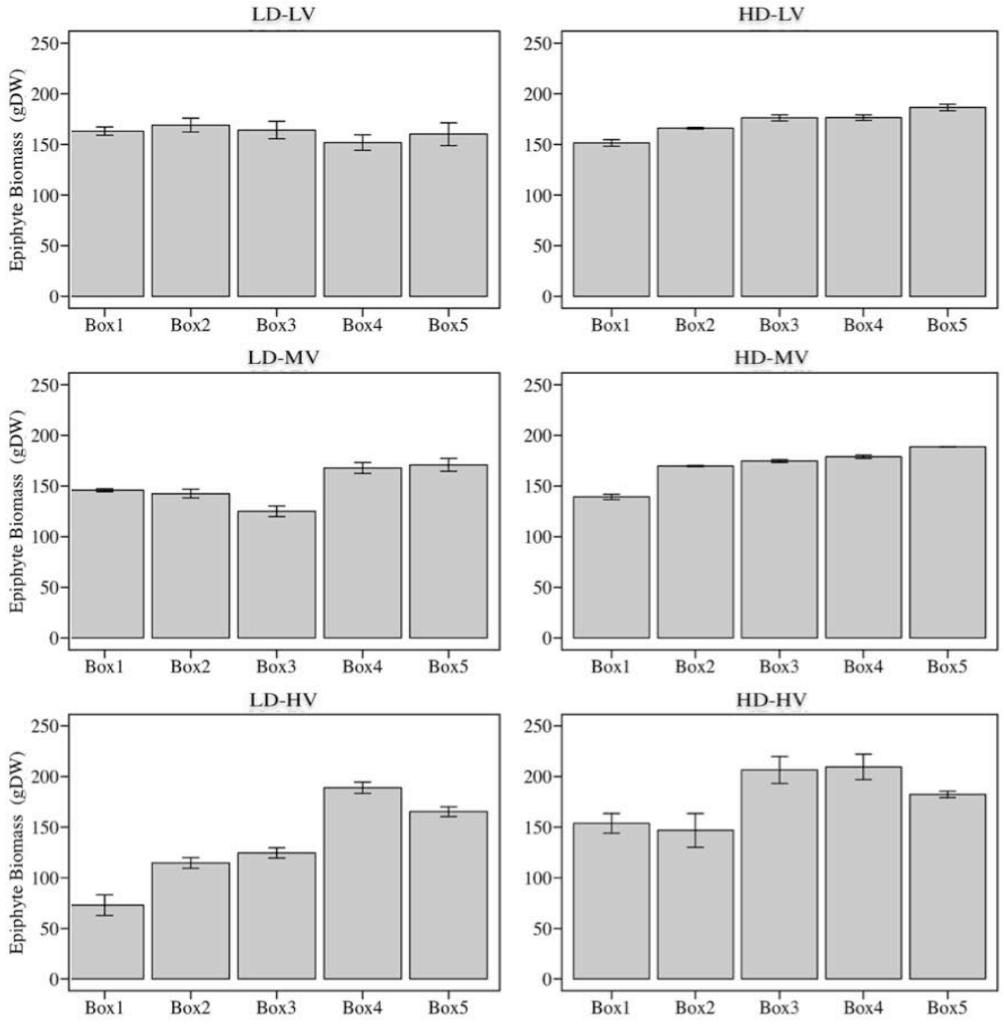

Figure 4. Mean ± s.e. (n = 3) of epiphyte load (g DW) in each box along the racetrack flume at the end of each run. Box 1 was placed at the leading edge of the artificial bed.

Table 3. Statistical results of the Kruskall Wallis test (High density) and 2-way ANOVA analysis (Low density) examining the effects of flow velocity and flume position (box identity) on epiphyte load in grazer and no-grazer treatments. Bold font indicates significance differences (p-value < 0.05)

Grazer treatments				
High density				
	df	**X^2**		***p***
Flow velocity	2	1.763		0.41
Flume position	4	30.918		**<0.0001**
Flow velocity-Flume position	14	38.609		**<0.0001**
Low Density				
	df	**MS**	**F**	***p***
Flow velocity	2	122.95	23.6	**<0.0001**
Flume position	4	119.85	23	**<0.0001**
Flow velocity-Flume position	8	87	16.7	**<0.0001**
Residuals	30	5.21		
No-grazer treatments				
High density				
	df	**X^2**		***p***
Flow velocity	2	0.128		0.93
Flume position	4	34.063		**<0.0001**
Flow velocity-Flume position	14	35.091		**0.0014**
Low Density				
	df	**MS**	**F**	***p***
Flow velocity	2	179.8	28.2	**<0.0001**
Flume position	4	176.9	27.7	**<0.0001**
Flow velocity-Flume position	8	85.8	13.5	**<0.0001**
Residuals	30	6.3		

Spatial position and swimming behaviour of Gammarus locusta

G. locusta location along the artificial bed was significantly affected by flow velocity, shoot density and time of observation (Figure5 and Table 4). In all the treatments, when individuals of *G. locusta* were released into the racetrack flume, they actively moved but the

effectiveness of their swimming movements depended on experimental conditions (Table 5). The swimming behaviour was most evident in treatments with low flow velocity (LV), since the amphipods tended to be distributed throughout the artificial bed (Figure5), especially under HD. In contrast, *G. locusta* usually showed limited mobility under HV treatments (Table 5) and tended to aggregate downstream of the leading edge (Figure5).

Figure 5. Three dimensional locations of *G. locusta* in the racetrack flume at four observational times- 10 min (•), 1h (▲), 3h (◆) and 6h (■). LD = Low density, HD = High density, LV = Low velocity, MV = Medium velocity, HV = High velocity.

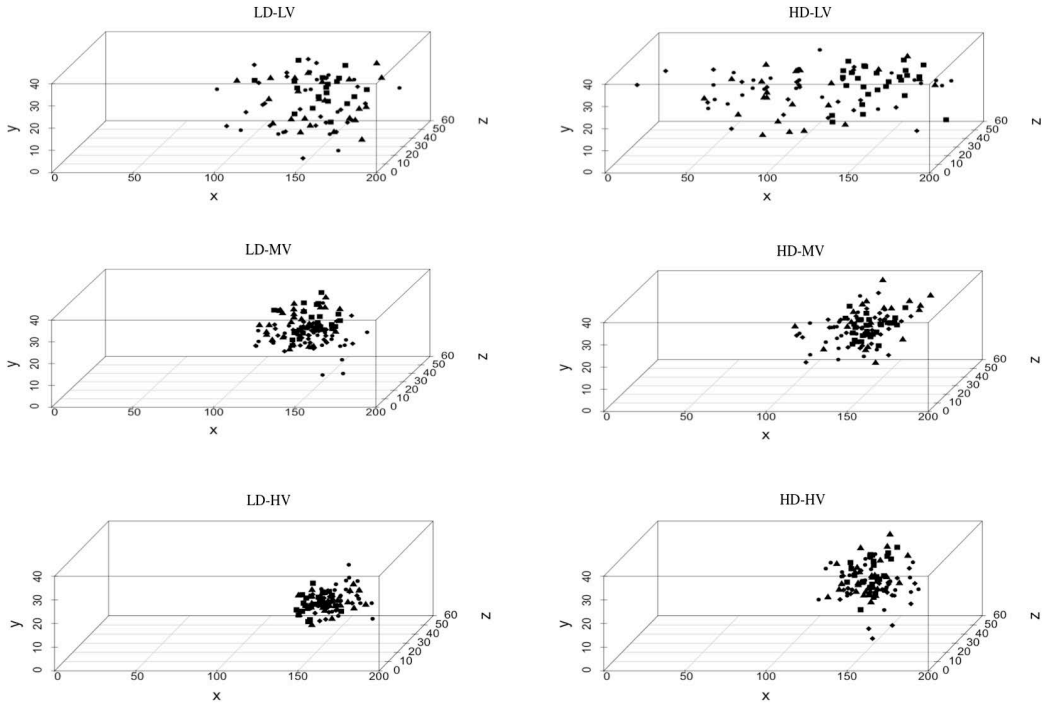

Table 4. Statistical results of the Kruskall Wallis test examining the effects of flow velocity and observation time on *G. locusta* positions along X, Y, Z axes. Bold font indicates significance differences (p-value < 0.05).

Low Density			
X	**df**	**X^2**	***p***
Flow velocity	2	33.2	**<0.001**
Time	3	15.38	**0.0015**
Flow velocity-Time	11	67.63	**<0.001**
Y			
Flow velocity	2	8.16	**<0.0001**
Time	3	21.78	**<0.0001**
Flow velocity-Time	11	52.51	**<0.0001**
Z			
Flow velocity	2	54.44	**<0.0001**
Time	3	12.93	**<0.0001**
Flow velocity-Time	11	89.21	**<0.0001**
High Density			
X	**df**	**X^2**	***p***
Flow velocity	2	44.28	**<0.001**
Time	3	20.91	**<0.001**
Flow velocity-Time	11	83.35	**<0.001**
Y			
Flow velocity	2	20.44	**<0.001**
Time	3	8.05	**0.044**
Flow velocity-Time	11	53.43	**<0.001**
Z			
Flow velocity	2	0.58	0.74
Time	3	7.91	**0.047**
Flow velocity-Time	11	14.92	0.18

Discussion

This flume experiment clearly indicates that unidirectional flow conditions, shoot density and their interactions significantly influenced the feeding and foraging behaviour of mesograzers in (artificial) seagrass meadows. This change in feeding and foraging behaviour also has major implications at the community level, affecting the density of epiphytes. Epiphyte consumption was very low both under high flow velocity conditions (HV) and at low density (LD). This was linked to changes in the behaviour of *G. locusta* (Figure5). That is, higher velocity resulted in shorter feeding time and lower swimming capacity, leading to lower rates of epiphyte removal. In addition, *G. locusta* tended to aggregate downstream of the leading edge of the reconstructed bed, where unidirectional flow velocity is highly reduced by the buffering capacity of the seagrass canopy (Bouma et al. 2005, Morris et al. 2008, González-Ortiz et al. 2014); thus, the epiphyte consumption was restricted to these discrete areas were grazers tended to aggregate. However, when hydrodynamic conditions were more gentle or shoot density was higher, the physical stress on the amphipods was lower. This allowed for investment in feeding and swimming (Pepper et al. 2015), leading to higher epiphyte removal from the canopy (Figure3).

Although the mimics do not offer a perfect model and do not perfectly reflect all the characteristics of the seagrass leaves (e.g. chemical properties or colour), they provide a way to homogenise and make similar replicates of experimental conditions. Moreover, previous flume tank experiments have demonstrated that the use of this kind of seagrass mimics replicates the main characteristics and interactions between unidirectional flow and seagrass canopy, and therefore are quite useful in this kind of approaches (Bouma et al. 2005). In addition, the pilot experiment demonstrated that *G. locusta* does not distinguish between epiphytes on natural seagrasses and those on mimics when feeding (Figure2). This indicates that using mimics with epiphytes is a valid and valuable experimental approach to address the mechanisms underlying epiphyte-grazer interactions; it allows for the development of treatments controlled for differences resulting from plant-epiphyte interactions (e.g. differences in leaf surface, leaf span time, natural products; Borowitzka and Lavery 2005, Teena et al. 2008).

Can we explain the epiphyte effects by changes in swimming behaviour?

Virtually all species of Gammaridean amphipods can swim, with some travelling long distances at a continuous rate, while others escape predators or change microhabitats with short bursts of rapid swimming (Boudrias 1991). In this experiment, we observed individuals of *G. locusta* to move quickly from mimic to mimic. At low velocity, individuals of *G. locusta* tended to swim and disperse across the flume in scattered positions (Figure5). In this scenario

of low velocity, especially under low density, higher percentages of swimming *G. locusta* were recorded (27.5± 4.18 % of individuals in the whole experimental time) compared to percentages of swimming for high density (18.35 ± 13.70 %). This result indicates that change in swimming behaviour and, thus, time spent in feeding, was responsible for the different rates of epiphyte removal.

Interacting feedback loops

Previous studies on the relationship between hydrodynamics and epiphyte-grazer systems found conflicting results on the effect of flow on epiphyte load. On the one hand, epiphyte load was found to be lower under high flow conditions because of the washing out of epiphytes,(e.g. physical erosion; Lavery et al. 2007). On the other hand, the washing out of grazers (i.e. *Hydrobia ulvae*) was found to be actually responsible for the increase in epiphyte load (Schanz *et al.* 2002). Our results agree with both perspectives, since the relationship between shoot density and flow velocity has demonstrated that outcomes are contingent on the strength of such interactions (Figure 6). For instance, high density may reduce physical stress for both epiphytes and mesograzers (a 1d model in Eckman 1983, Lavery et al. 2007, this work, Table 3 and Figure 4), reducing both the washing out of epiphytes and predation pressure on mesograzers while increasing the feeding time (Gilliam and Fraser 1987, Schanz et al. 2002, Borowitzka and Lavery 2005, Lavery et al. 2007, González-Ortiz et al. 2014, this work). In addition, high habitat complexity reduces light and nutrient availability for epiphytes (Lavery *et al.* 2007; Morris *et al.* 2008, 2013), which may decrease their occurrence frequency in the canopy. High flow velocity may increase nutrient transfer to epiphytes (Morris *et al.* 2008, 2013) while increasing self-shading because of the higher bowing of the canopy (Morris *et al.* 2008; González-Ortiz *et al.* 2014b), which in turn reduces light availability (Drake *et al.* 2003). Also, as demonstrated in this work when using an unidirectional flow, amphipods can be physically washed away from some locations (e.g. leading edge of the patch) where higher flow velocity and turbulence values are expected (Morris *et al.* 2008; González-Ortiz *et al.* 2014b). Most of these interrelationship paths are bidirectional and also affect habitat complexity (e.g. shoot density, leaf length, above/belowground ratio; Brun et al. 2006). Finally, these paths may affect ecosystem properties, such as the transfer of matter and energy to higher trophic levels (Duffy *et al.* 2005; Heck *et al.* 2008; Poore *et al.* 2012) (Figure6). However, caution should be taking when considering trophic cascade effects, since it may imply at least another level above the herbivores, which is not contemplated in this experimental approach.

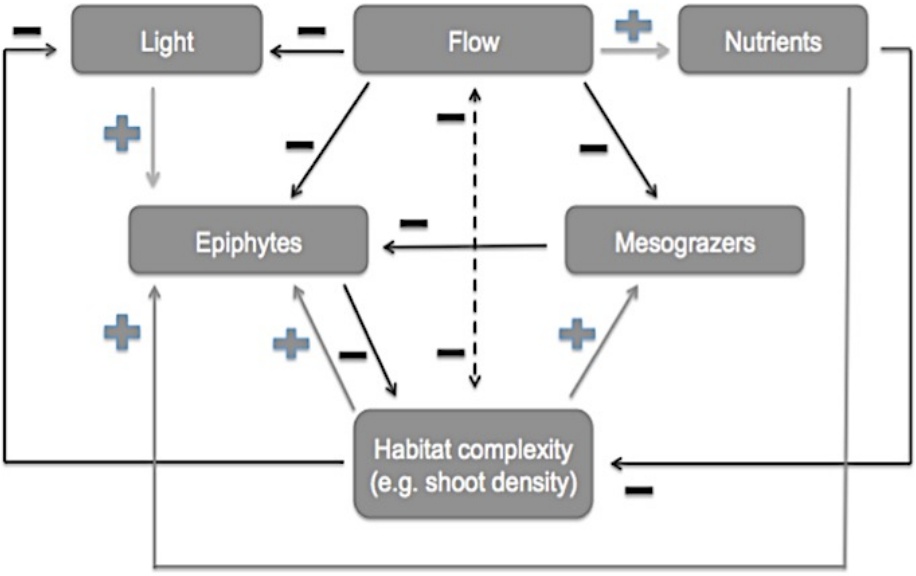

Figure 6. Conceptual diagram showing the positive or negative relationships among flow velocity, habitat complexity, and biotic and abiotic factors. Black lines indicate negative relations. Grey lines indicate positive relations. Dashed line shows bidirectional relationship.

Ecosystem implications

Recent studies have recognized that eutrophication is a widespread disturbance that can severely impact coastal areas, including seagrass ecosystems, through several mechanisms, such as decreasing light availability by promoting macroalgae and epiphytic growth (e.g. Waycott et al. 2009). However, epiphyte removal by grazers has been found to enhance productivity and biomass of seagrasses and to buffer the negative consequences of eutrophication (Hootsmans and Vermaat 1985; Howard and Short 1986; Philippart 1995; Nelson and Waaland 1997; Duffy et al. 2005; Moksnes et al. 2008; Poore et al. 2012). This study clearly indicates that seagrasses act as foundation species, having a positive biological effect on mesograzers through facilitation (i.e. reducing hydrodynamic pressure). Not only does reducing hydrodynamic pressure have a direct impact on mesograzers, it also forms a feedback to the foundation species by reducing epiphyte load. By removing epiphyte biomass, grazers can substantially impact the persistence of seagrasses by promoting trophic cascade effects, thus stimulating the ecosystem services and functions of these foundation species (Klumpp et al. 1992; Philippart 1995; Duffy and Harvilicz 2001; Moksnes et al. 2008; Poore et al. 2012).

Conclusions

Our results clearly demonstrate that the triggering of trophic cascades depends on the strength of the interrelationships and feedbacks among habitat complexity, abiotic and biotic compartments, with flow velocity acting as a top-down regulator (*sensu* Schanz et al. 2002) in seagrass ecosystems. Therefore, these complex relationships must be considered in such habitats, since it may determine conditional outcomes at the level of communities and ecosystems. Furthermore, seasonal and spatial effects on these relationships should be addressed, since both are known to affect seagrass habitat complexity, hydrodynamic conditions and biotic compartments.

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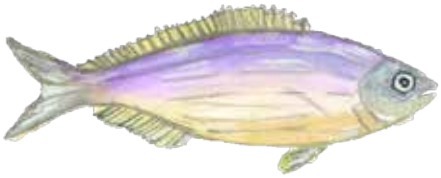

CHAPTER 2

Hydrodynamics as a decisive factor constraining *Cymodocea nodosa* defoliation by consumers

In preparation

En la vida no hay nada que temer, solo hay que comprender.
Nothing in life is to be feared, it is only to be understood.

Marie Curie

Hydrodynamics as a decisive factor constraining *Cymodocea nodosa* defoliation by consumers

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Abstract

Defoliation rates of *Cymodocea nodosa* plants were evaluated in a annual cycle, in four locations characterized by contrasting hydrodynamic conditions, to study how hydrodynamics alter leaf palatability and grazers activity, and thus herbivory rates. Cross-transplantation experiments among each area were carried out once per season, to encompass the seasonal variation in hydrodynamic conditions, density of consumers and changes in leaf traits. Moreover, in each location, leaf properties, annual seagrass production and abundance of grazers were measured. A negative relationship between defoliation rates and hydrodynamic conditions were recorded, with the highest rates of annual defoliation at the location with mild hydrodynamic conditions (74% of annual production), and lower values at the most exposed (46.5 % of annual production). Leaf traits also differed among locations and seasons recording the most palatable plants in sheltered areas. In this location, a significant reduction of plant defoliation in those shoots transplanted with respect to native ones was recorded. This results was probably due to the lack of physical stress over the consumers, and thus they can invest more energy in swimming and selecting those leaves more palatable. Hence, our results suggest that herbivory in seagrass communities is largely controlled by hydrodynamic conditions because of reducing grazer activity and altering leaves palatability.

Introduction

Seagrasses are considered as foundation species hosting high biodiversity levels in coastal areas (Heck et al. 2003), but little attention has been given to the seagrass-herbivore interactions in past studies (Kikuchi and Peres 1977; Thayer et al. 1984). However, it is increasingly recognized that seagrass-associated organisms, as well as seagrass herbivores can influence the structure of seagrass populations, while seagrass grazers may play a key role in the seagrass food webs (Mariani and Alcoverro 1999; Alcoverro and Mariani 2004; Valentine and Duffy 2006; Prado et al. 2007). There are a wide variety of seagrass grazers, from mesograzers to large vertebrates (see Heck and Valentine 2006 and references therein), which are known to feed selectively, influenced by leaf nutritional quality (i.e. nitrogen content; McGlathery 1995, Cebrián and Lartigue 2004, Goecker et al. 2005), as well as by structural and chemical defenses (i.e. carbon and fiber content or phenolic compounds; Vergés et al. 2007a, b, Prado and Heck 2011). Such leaf traits determine seagrass palatability, which can be defined as plant susceptibility to be consumed (Heady and Child 1994). Leaf palatability is modified by several abiotic factors such as nutrients content in seawater (Tomas et al. 2011), temperature or acidification events (Poore et al. 2013), which modify their consumption. In addition to aforementioned factors, hydrodynamics also contribute to modify seagrass palatability, since plants acclimate to such abiotic factor by tuning their morphological and structural traits (De Los Santos et al. 2012, 2013, 2016), and such traits have been commonly reported as an effective anti-herbivore defense in terrestrial ecology (Wright and Vicent 1996; Pennings et al. 1998; Lucas 2000; Lanyon and Sanson 2006). Therefore, seagrasses when are exposed to high hydrodynamic levels enhance their mechanical resistance, and it limits the ability of the herbivores to shear or tear the leaves for their intake (Read and Stokes 2006; de los Santos et al 2013; Martínez-Crego et al. 2016).

On the other hand, hydrodynamics may also directly influence on the composition of consumers, and even the physical stress imposed by hydrodynamics may modify the feeding and foraging behaviour of grazers (Schanz et al. 2002, Jiménez-Ramos et al, submitted), and therefore may control the consumption of seagrass leaves. In order to demonstrate experimentally this main goal, two secondary goals were established: 1) to evaluate if the acclimation to environmental conditions determines seagrass palatability and 2) to determine if the abiotic conditions (e.g. hydrodynamics) regulate patterns of consumption of seagrass leaves.

To do that, an annual cross-transplantation experiment was carried out in four locations in Cádiz Bay (southern Spain) with contrasting hydrodynamic conditions inhabited by the template seagrass *Cymodocea nodosa*. This bay is an excellent outdoor laboratory to undertake this study, since the seagrass *C. nodosa* inhabit several locations with different degrees of exposure to currents and waves, showing contrasting leaf traits among locations (Brun et al. 2003a; Brun et al. 2005; de los Santos et al., 2013)

Material and methods

Study site

In situ experiments were conducted in *C. nodosa* beds, which form perennial populations in Cadiz Bay (south of Spain) in locations with different abiotic conditions: Caño de Cortadura (CC), Santibáñez (ST), Bajo de la Cabezuela (BC), El Chato (CH) (Figure 1). The "Caño de Cortadura" (CC, 36º 52' 60"N – 06º 21' 77"W) is a small and shallow lagoon located near the Rio San Pedro, connected to the San Pedro river through a small artificial channel. This is a sheltered location with mild hydrodynamic conditions and without waves, influenced only by semidiurnal tidal oscillations. The most common consumers of *C. nodosa* plants in this location are the amphipod *Gammarus* sp., the isopod *Idotea* sp. and *Lekanesphaera hookeri* (personal observation).

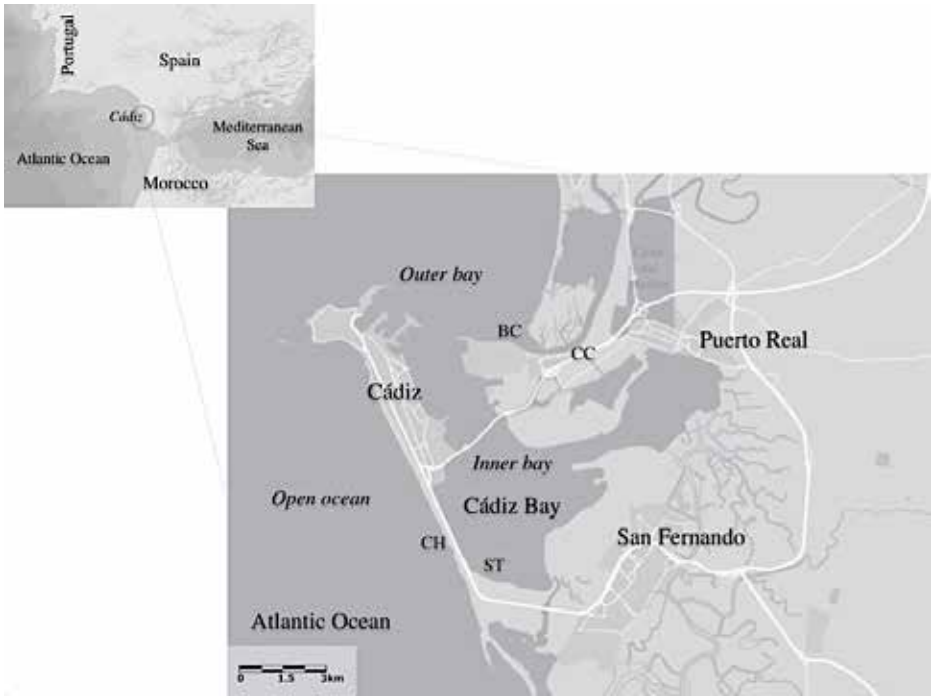

Figure 1. Map of the study area in SW Spain showing the location of *Cymodocea nodosa* beds at each study sites (from low to high exposure, CC: Caño Cortadura; ST: Santibáñez; BC: Bajo de la Cabezuela; CH: El Chato).

Santibáñez saltmarsh (ST, 36º 28' 09.08"N – 06º 15' 04.64"W) is located in the south point of the inner bay, a shallow tidal lagoon (3m mean depth with respect to the mean low water) with wide tidal flats and complex system of tidal creeks. This is a site of medium hydrodynamics, with little exposure to waves. The mean velocity module during a tidal cycle ranges from 0.015 to 0.08 m/s (Lara et al. 2012), which is expected to increase when strong eastern winds blow, and during winter time (Kagan et al. 2003). *C. nodosa* populations inhabits the shallow south-western area at both the intertidal fringe and as continuos monospecific meadows at the subtidal zone, at 0.4 and -0.5 m above the chart datum (lowest astronomical tide), respectively (Olivé et al. 2013). *C. nodosa* populations in ST exhibit an unimodal seasonal pattern of growth, with maximum growth rates and productivity in summer (Egea et al, submitted). The shoot density and biomass in this area vary with tidal position (de los Santos et al. 2013). The decapod *Hyppolite* sp. and the amphipod *Gammarus* sp. are the main mesograzers in this location, and the maximum abundance is at the beginning of summer (López de La Rosa et al. 2006).

The Bajo de la Cabezuela (BC, 36º 31' 42.52"N – 06º 14' 32.16"W) is a shallow inlet located at the Rio San Pedro saltmarsh and comprises a tidal sound with its wider mouth opened to outer Cadiz Bay and the other artificially closed upstream. This sandflat is characterised by semidiurnal mesotides with the tidal current flowing from the bay along the inlet. Plants were collected at the mouth of the inlet, a location subjected to high currents powered by tide, and short-period wind-generated waves (which increased during the winter), and recurrent episodes of sediment erosion and acretion (Kagan et al. 2003; Brun et al. 2005). Mean velocity module measured 10 cm above the sea floor during a tidal cycle ranged from 0.05 to 0.25 m/s (Lara et al. 2012).

In the last two locations (ST and BC), the fish *Sarpa salpa* is commonly seen feeding at depths between 1 and 15 m, particularly during the warm period (i.e. late June to late September; Crespo and Ponce 2003). Therefore, the present study was undertaken within the depth range of maximum herbivore density (i.e. 1 to 5 m depth in high tide), where herbivores can potentially have a relevant effect on the local communities of *C. nodosa*.

El Chato (CH, 36º28'38.16"N-06º15'49.21"W) is a shoreline parallel rocky outcrop located at the Cortadura beach, facing the open ocean with a NNW–SSE orientation. The rocky platform has a maximum width of 100–200 m (1 to 1.5 above chart datum) and presents several intertidal pools with sandy accumulation on bottom (medium sand; averaged depth of 0.20 ± 0.10 m; Velasco 2006). The hydrodynamic conditions of this location highly differ from the others as it is the only location exposed to open ocean, thus exposed to strong currents (data not available) and large waves reaching and breaking on the rocks (mean annual significant wave height 1.13 m and maximum of 5.40 m; Puertos del Estado 2009). *C. nodosa* forms small scattered age of low shoot density in the rocky pools. The potential consumer in this location is the sea urchin *Paracentrotus lividus* (personal observation). Sampling was performed seasonally throughout the year, to encompass the seasonal variation in hydrodynamic conditions and densities of consumers.

Leaf traits analyses

For each location, 5 samples were haphazardly chosen with a 400-cm^2 quadrat to determine seasonally the shoot density. Moreover, morphology (length, width and thickness), nutritional (nitrogen content), structural (carbon and fiber content) and biomechanical (force-to-cut; FTA and FTS) traits were examined in ten independent shoots in each quadrat (n=10). Prior to measurements, seagrass leaves were cleaned of epiphytes carefully using a piece of soft paper. Total leaf length was measured with a ruler (accuracy 1.0 mm), and leaf width and thickness with a digital caliper and a dial thickness gauge, respectively (accuracy 0.01 mm; Mitutoyo®). The water content in the samples

was calculated as the weigh difference between fresh and dried biomass after 48 hours in the oven (60ºC). Samples were freeze-dried and pulverized in a ball-grinder to determine the total C and N content in tissues and fibre content. Elemental analysis was performed in a Perkin-Elmer 2400 elemental analyzer, while fibre content was done using the method of Van Soest et al. (1991) modified by De Los Santos et al. (2012).

The biomechanical traits of *C. nodosa* leaves were measured with an Instron testing machine (model 5542) and BlueHill® software (v.2.18) in ten shoots per location. We sampled the first outermost fully-developed leaf from the selected experimental plant units (EPUs, normally the second youngest leaf), and a portion of the leaf blade was cut 4 to 5 cm above the ligule for testing. The leaves were measured within 1d of sampling and the specimens were tested in the same sequence as they had been collected so that the time of storage was similar among samples and treatments. Leaf-fracture traits were evaluated by cutting tests. The cutting test measures the force required for foliar breakage (Wright and Vicent 1996; Aranwela et al. 1999). During the test, a force to displacement curve was monitored. Since the whole leaf was cut transversally during the test, the force exerted to cut the lamina also included the leaf veins (De Los Santos et al. 2012). Results were expressed at 2 levels: (1) total quantity of force needed to cut or tear a single leaf blade, which depends on the leaf size and its mechanical traits at the material level (FTA, N); (2) material biomechanical traits, normally called 'material properties', which are inherent traits of the material (FTS; N mm^{-2}).

Epifauna collection samples and processing

Triplicated benthic and epifaunal samples were collected in the four-study location using acrylic cores (20 cm diameter and 50 cm long) close to the points where samples for shoots density were collected. A 1 mm mesh bag was attached to one end of the tube, and the other end slipped over the seagrass patch and onto the sediment. Seagrass leaves were cut off at the base with a scissor, and the content (leaves and epifauna) were collected into the mesh bag (see Douglass et al. 2010). Epifaunal samples were frozen at -20ºC until sorting. During sorting, seagrass leaves were separated from fauna and benthic/epi-fauna were identified to the lowest taxonomic level, and subsequently sorted in function of their feeding behaviour.

Sea urchins abundance was measured in the field using a 1 m^2 quadrat at 10 different selected randomly areas into the *Cymodocea* beds from CH. There is a lack of information on abundance values of the *Salpa sarpa* in the Cádiz bay. According to the existing literature, its maximum abundance is between spring and summer (Arias and Drake 1990).

C. nodosa defoliation and productivity

A tethering experiment (Kirsch et al. 2002; Prado et al. 2007) was conducted to obtain direct quantification of *C. nodosa* defoliation by herbivores. At each location, we collected *C. nodosa* shoots haphazardly at the same depth (intertidal area). Care was taken to collect shoots with intact vertical rhizomes to minimize changes in the leaves properties and in order to allow the safeguarding of leaves throughout the experiment. Once in the laboratory and prior to any measurement, 40 shoots of each location were carefully selected according to its healthy appearance (no necrosis or bites) and epiphytes were scraped carefully. After that, the number of leaves and their length were recorded. Experimental shoots were marked using a modified Zieman method (Zieman 1974; Peralta et al. 2000) in order to detect possible leaf elongation during the experiment and to measure initial leaf lenght.

In order to optimize retrieval of samples, individual shoots were attached by their vertical rhizome to a line by a bowline knot and randomly, and each line carrying a total of 2 labelled pickets. In order to determine whether the *C. nodosa* consumption is mediated by the location or by the leaf properties, in each line was attached a shoot from the different location and were separated from each other by approximately 0.25 m (i.e. each line hold 4 shoots) (Figure 2). Ten lines were set up inside the meadow at each location at the same depth of collection (equally for both all sites, 1.5 m approximately in low tide), lines were placed approximately one after the other, separated by 3 meters, and, thus, each line was considered an independent sample. Shoots were attached in lines randomly. Therefore, each line contains a native shoot with three shoots from remaining experimental sites. During laboratory and field manipulation and transport, plants were kept underwater to avoid stress. Moreover, when inserting pickets into the sediment, maximum care was taken to ensure that leaves were neither above nor below the average height of the leaf canopy.

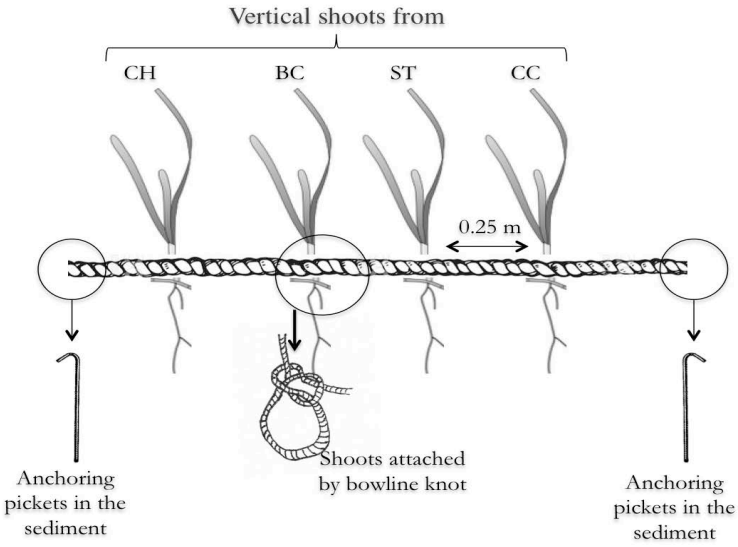

Figure 2. Drawing of experimental set-up for tethered method to measure defoliation rates in *C. nodosa* shoots. Shoots were attached randomly.

After 2 weeks tethered shoots from four-study location (CH, BC, ST, CC) were carefully collected and cold transported. Once in the laboratory, leaf length and width, the number of leaves per shoot that were lost, those that were still intact, or were attacked by grazers, were recorded. Herbivore leaf bite marks were divided in 3 identifiable shapes respect to the physiology of their oral apparatus: bites from fishes (semicircular shape), bites from browsed mesograzers (not identifiable between species of mesograzers) and sea urchin (irregularly slashed scars and only present in the CH) (see Figure 3).

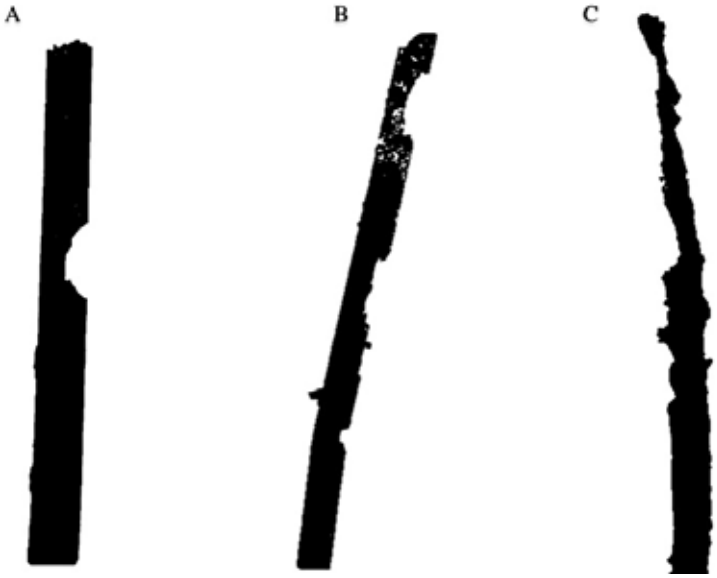

Figure 3. Real images of *C. nodosa* leaves from the experimental set-up with morphologically distinctive bite marks of: A. *Sarpa salpa*, B. Mesograzers and C. *Paracentrotus lividus*.

Leaf elongation rate (cm^2 $shoot^{-1}$ d^{-1}) of tethered shoots was calculated by dividing the area of new tissue produced by the number of days elapsed since marking (i.e. 15d). Leaf elongation rate allows to correct the defoliation estimates. Seagrass defoliation was estimated by subtracting the leaf area remaining from each initial shoot measurement and by adding the growth area of the tethered shoots obtained after 2 weeks, and were expressed in cm^2 $shoot^{-1}$ d^{-1} (Kirsch et al. 2002). Leaves that were lost during this period were not included in the analyses, which may cause an underestimation of the total defoliation rates.

On the other hand, leaf productivity of *C. nodosa* at the four-study locations were measured using a "punching method" (Peralta et al. 2000) in 10 independent uprooted shoots (called UPS) seasonally. Rates of leaf production for each seasonal period were expressed in terms of cm^2 $shoot^{-1}$ d^{-1} (i.e. leaf elongation) and then compared to seagrass defoliation obtained in tethered shoots.

Statistical analyses

Previously, homogeneity of variances (Levene's test) and normality (Shapiro–Wilks test) were tested. Differences in leaf traits, annual productivity and annual defoliation at each

location was assessed by Kruskal-Wallis test followed by post hoc multiple comparisons (Tukey HSD). An effect size meta- analysis was also run to determinate the differences in defoliation rates by consumers between native *vs* cross-transplanted plants in each location yearly in order to avoid the possibly misleading influences of sample size. Effect size and its confidence interval enable to make more biologically relevant decisions because allows an effective statistical inference from data, offering a better understanding and characterization of the results (Nakagawa and Cuthill 2007). While null hypothesis significance testing only informs about the probability of an observation, the presentation of effect size along with its standard error (s.e.) provides the two most important pieces of statistical information for biologists: the magnitude estimate of an effect of interest and the precision of that estimate (Nakagawa and Cuthill 2007). Thus, if there are non-significant differences but large effects, it may suggest further research with greater power (Fritz et al. 2012). To estimate the effect size of the parameters under study we chose the Hedges' d metric (Hedges et al. 1985) because it is an unbiased estimator that provides a better estimate for small sample sizes. Effect size is presented as Hedges' d ± standard error (s.e.). Hedges' d metric values above 0 indicate a positive effect, bellow 0 indicate a negative effect, and equal to 0 indicates no effect on the parameter under investigation. The bigger the number either on the positive or negative direction tells about the magnitude of the effect. Cohen (1988) has proposed 'conventional' values as benchmarks for what are considered to be 'small', 'medium', and 'large' magnitude of the effects (d = 0.2, 0.5, 0.8, respectively) (Nakagawa and Cuthill 2007).

Results

Spatial and temporal differences in leaf traits

On an annual basis, shoot density was higher in *C. nodosa* meadows from ST, obtaining the maximum values in summer (630.12 ± 29.12 shoot $\cdot m^{-2}$). On the other hand, meadows from the CH attaining the lower shoot density, reaching minimum values in winter (25 ± 7.69 shoot $\cdot m^{-2}$) (Table 1).

Leaf traits showed spatial and temporal variability showing higher nutritional traits (i.e. high nitrogen content) those plants coming from locations with lower hydrodynamic conditions (CC and ST), while higher structural traits (i.e. high carbon and fiber content) were recorded in those locations more exposed (BC and CH). Otherwise, the thinnest and shortest leaves were found in the CH, which also bear the highest structural traits (%C, NDF, FTS). Plants from BC had the most resistant leaves (i.e. higher FTA and FTS) in spring (Figure 4, Table 2).

Table 1. Above- and belowground biomass (g DWm^{-2}) and shoot density (shoot m^{-2}) of *C. nodosa* meadows from the 4 experimental locations (from low to high exposure, CC: Caño Cortadura; ST: Santibáñez; BC: Bajo de la Cabezuela; CH: El Chato) at each sampling time. Data are showed as mean ± s.e. (n=5)

	Aboveground biomass (gDW m^{-2})	**Belowground biomass (gDW m^{-2})**	**Density (shoot m^{-2} d^{-1})**
Autumn 2015			
CC	65.76 ± 14.64	82.16 ± 21.12	161.53 ± 22.67
ST	109.4 ± 10.59	195.16 ± 50.51	410.25 ± 63.61
BC	77.6 ± 11.60	328.93 ± 18.69	80.12 ± 20.56
CH	20.9 ± 7.18	46.23 ± 10.36	48.07 ± 26.43
Winter 2016			
CC	66.23 ± 10.83	89 ± 10.88	140.38 ± 23.39
ST	78.2 ± 10.54	188.1 ± 11.45	280.12 ± 17.44
BC	55.26 ± 6.55	321.03 ± 22.51	80.12 ± 28.93
CH	12.71 ± 4.06	26.13 ± 5.08	25 ± 7.69
Spring 2016			
CC	141.23 ± 16.41	99.33 ± 10.81	351.28 ± 54.53
ST	148.53 ± 15.90	144.3 ± 9.20	577.56 ± 30.84
BC	93.73 ± 15.27	185.06 ± 53.69	183.33 ± 11.59
CH	33.73 ± 6.69	45.46 ± 9.15	125.64 ± 14.93
Summer 2016			
CC	98.22 ± 8.95	111.63 ± 4.99	316.66 ± 87.90
ST	97.9 ± 9.41	83.86 ± 23.62	630.12 ±29.12
BC	68.46 ± 14.68	71.87 ± 20.83	219.87 ± 94.06
CH	49.2 ± 13.57	44.1 ± 3.65	108.97 ± 35.78
Autumn 2016			
CC	64.6 ± 12.2	71.78 ± 12.10	187.17 ± 21.87
ST	104.36 ± 3.88	158.18 ± 46.73	351.28 ± 25.09
BC	61.83 ± 13.68	257.36 ± 50.29	118.58 ± 8.67
CH	31.13 ± 7.44	48.53 ± 18.25	96.15 ± 39.45

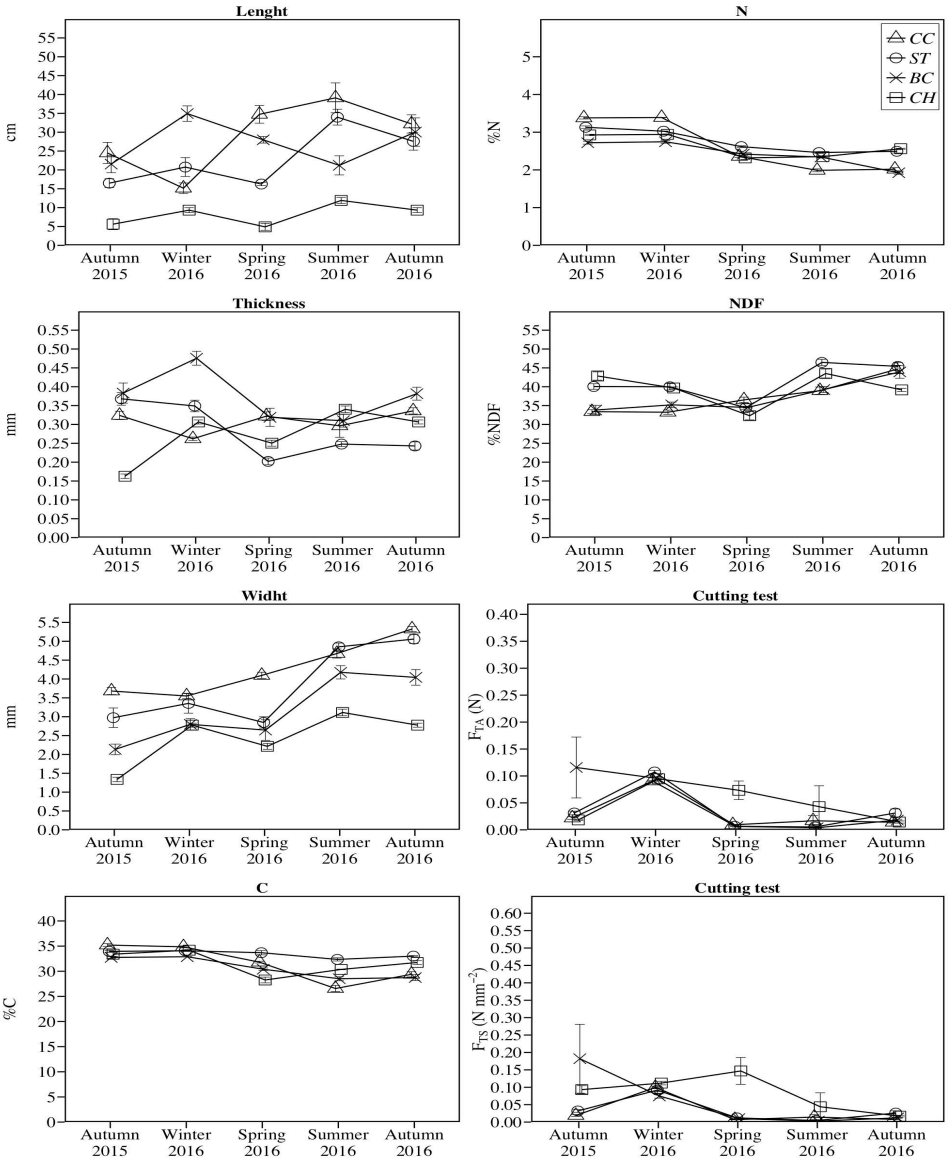

Figure 4. Spatial-temporal differences in the morphological, nutritional, structural and biomechanical traits of *C. nodosa* leaves from the four experimental locations (from low to high exposure, CC: Caño Cortadura; ST: Santibáñez; BC: Bajo de la Cabezuela; CH: El Chato) at each sampling time. Length (cm); Thickness (mm); Width (mm) for morphometry; Nitrogen content in leaves (%N) for nutritional traits; Carbon content (%C) and fiber content (%NDF) in leaves for structural traits; Absolute force-to-cut (FTA, N) and specific force-to-cut (FTS, N $\cdot$mm^{-2}) for biomechanical traits.

Table 2. Statistical results of the Kruskall Wallis test examining leaf traits at each sampling site. Bold font indicates significance differences ($p<0.05$).

	df	X^2	*p*-value
Lenght			
Season	4	12.106	**0.016**
Location	3	108.59	**<0.01**
Season:Location	19	152.24	**<0.01**
Thickness			
Season	4	14.507	**0.0058**
Location	3	40.953	**<0.01**
Season:Location	19	140.03	**<0.01**
Width			
Season	4	74.375	**<0.01**
Location	3	74.949	**<0.01**
Season:Location	19	164.93	**<0.01**
N			
Season	4	142.21	**<0.01**
Location	3	16.329	**<0.01**
Season:Location	19	188.67	**<0.01**
C			
Season	4	105.34	**<0.01**
Location	3	33.387	**<0.01**
Season:Location	19	172.18	**<0.01**
NDF			
Season	4	80.9	**<0.01**
Location	3	23.072	**<0.01**
Season:Location	19	155.04	**<0.01**
FTA			
Season	4	113.75	**<0.01**
Location	3	5.077	0.166
Season:Location	19	140.66	**<0.01**
FTS			
Season	4	100.48	**<0.01**
Location	3	22.415	**<0.01**
Season:Location	19	137.81	**<0.01**

Fauna samples

A total of 7 taxa were found in all sample sites (including Decapoda, Amphipoda, Isopoda, Equinodermata, Gastropoda, Polyplacophora, Bivalvia and Polychaeta) (Table 3). The most abundant species found in the CC were the decapod *Hyppolyte* sp., the amphipod *Gammarus* sp. and the isopods *Idotea* sp. and *Lekanesphaera hookeri* with higher abundances in Spring and Summer. A high abundance of *Hyppolyte* sp., was also found in ST, especially in summer (3769 ± 1543 ind·m^{-2}) and a lower abundance in BC (805 ± 326 ind·m^{-2}) joined with the other decapod *Palaemon adspersus*. The equinodermata *Paracentrotus lividus* was found only in the CH with a great presence especially in Spring (30 ± 6 ind·m^{-2}). Otherwise, we did not directly quantified *Salpa sarpa*, but it is commonly observed in the ST and BC area, especially during the summer (Arias and Drake 1990; Crespo and Ponce 2003).

Table 3. Abundance of species (ind·m^{-2}) found at each sampling sites in each season yearly. C: carnivore; D: feeds on detritus; DF: deposit feeder; H: herbivore; O: omnivore; SF: suspension feeder. Data are showed as mean ± s.e. (n=3)

Taxa	Epi-fauna	Feeding behaviour	CC Autumn 2015	ST Autumn 2015	BC Autumn 2015	CH Autumn 2015	CC Winter 2016	ST Winter 2016	BC Winter 2016	CH Winter 2016	CC Spring 2016	ST Spring 2016	BC Spring 2016	CH Spring 2016	CC Summer 2016	ST Summer 2016	BC Summer 2016	CH Summer 2016	CC Autumn 2016	ST Autumn 2016	BC Autumn 2016	CH Autumn 2016
Decapoda	*Palaemon serratus*	O	0	25 ± 11	0	33 ± 12	0	0	0	15 ± 9	0	2 ± 4	0	86 ± 14	0	0	0	150 ± 35	0	3 ± 5	0	56 ± 31
Decapoda	*Hippolyte sp.*	O	547 ± 134	834 ± 145	342 ± 125	0	85 ± 12	134 ± 76	99 ± 32	0	759 ± 234	994 ± 328	327 ± 45	0	854 ± 265	3769 ± 1543	805 ± 326	0	481 ± 84	943 ± 47	165 ± 114	0
Decapoda	*Palaemon adspersus*	O	0	54 ± 32	26 ± 8	19 ± 22	0	69 ± 45	6 ± 4	3 ± 5	0	189 ± 68	26 ± 9	34 ± 27	0	215 ± 89	28 ± 16	49 ± 17	0	60 ± 53	44 ± 19	31 ± 22
Amphipoda	*Gammarus sp.*	D, H	329 ± 51	234 ± 108	6 ± 5	0	67 ± 78	44 ± 26	0	0	452 ± 123	556 ± 95	8 ± 4	0	537 ± 2231	779 ± 12	24 ± 15	0	267 ± 89	121 ± 41	8 ± 5	0
Isopoda	*Idotea sp.*	D, H	94 ± 56	0	0	0	32 ± 10	0	0	0	89 ± 31	19 ± 8	0	0	77 ± 18	27 ± 22	0	0	56 ± 27	7 ± 6	0	0
Isopoda	*Lekanesphaera hookeri*	D, H	34 ± 25	3 ± 5		0	16 ± 9	0	0	0	33 ± 24	0	0	0	21 ± 12	0	0	0	76 ± 54	0	0	0
Sparidae*	*Salpa sarpa**	O	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	Benthic fauna																					
Equinodermata	*Paracentrotus lividus*	O	0	0	0	4 ± 3	0	0	0	0	0	0	0	30 ± 6	0	0	0	10 ± 3	0	0	0	7 ± 4
Gastropoda	*Bulla striata*	C	5 ± 2	3 ± 5	0	0	0	0	0	0	0	8 ± 9	4 ± 6	0	0	1 ± 3	0	0	0	0	0	0
Gastropoda	*Calyptrea chinensis*	SF	0	1 ± 2	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Gastropoda	*Littorina sp.*	DF	0	0	0	0	0	0	0	0	3 ± 2	0	0	0	5 ±3	0	0	0	0	0	0	0
Gastropoda	*Natica sp.*	C	0	0	0	0	0	0	0	0	0	0	2 ± 3	0	0	0	0	0	0	0	0	0
Gastropoda	*Nassarius sp.*	O	0	0	0	6 ± 3	0	0	0	0	0	2 ± 4	0	0	0	1 ± 0.5	0	8 ± 6	0	0	0	0
Gastropoda	*Nassarius reticulatus*	O	0	0	0	0	0	0	0	0	0	0	1 ± 0.5	0	0	0	0	0	0	0	0	0
Gastropoda	*Turritela sp.*	DF	3 ± 1	0	6 ± 3	4 ± 2	0	0	0	3 ± 5	0	0	10 ± 8	7 ± 2	0	0	6 ± 3	15 ± 7	0	0	0	0
Gastropoda	*Hydrobia ulvae*	DF	0	45 ± 12	0	0	0	0	0	0	0	58 ± 34	0	0	0	69 ± 25	0	0	0	17 ± 9	0	0
Polyplacophora	*Chiton sp.*	H	0	0	0	0	0	0	0	0	0	0	0	0	0	2 ± 0.5	0	0	0	0	0	0
Bivalvia	*Loripes lucinalis*	SF	0	0	8 ± 4	0	0	0	0	0	5 ± 1	6 ± 3	12 ± 4	0	0	13 ± 9	19 ± 8	0	0	0	12 ± 5	0
Bivalvia	*Paphia aurea*	SF		0	7 ± 3	0	0	0	0	0	0		0	0	0	0	8 ± 6	0	0	0	4 ± 0.5	0
Bivalvia	*Solen marginatus*	SF	0	0	0	0	0	0	0	0	0	0	8 ± 6	0	0	0	4 ± 5	0	0	0	0	0
Bivalvia	*Macoma melo*	SF	0	23 ± 5	16 ± 10	0	0	5 ± 3	0	0	0	4 ± 1	6 ± 4	0	0	15 ± 5	25 ± 8	0	0	7 ± 4	16 ± 2	0
Bivalvia	*Venerupis philippinarum*	SF	0	12 ± 9	5 ± 2	0	0	0	0	0	0	2 ± 0.5	13 ± 3	0	0	18 ± 6	20 ± 2	0	0	3 ± 0.5	26 ± 7	0
Bivalvia	*Tellina sp.*	DF	0	0	0	0	0	0	0	0	0	0	2 ± 0.5	5 ± 2	0	0	0	0	0	0	0	0
Polychaeta	*Nereididae sp.*	O	5 ± 3	3 ± 0.5	0	0	6 ± 1	2 ± 3	0	0	5 ± 2	7 ± 4	3 ± 2	0	4 ± 1	5 ± 4	1 ± 0.2	0	2 ± 0.3	0	0	0

C. nodosa defoliation and production

The mean annual production differed among locations and seasons (Table 4, 5) showing CC and ST the higher C. nodosa production (Table 4). Annual defoliation rates of C. nodosa plants were estimated in each experimental site (CC, ST, BC, CH) for each UPS. Considering only the UPS, the % of annual defoliation respect to the annual production of C. nodosa was calculated in each location. The patterns varied among locations and were inversely related to hydrodynamic conditions, obtaining the maximum values of annual defoliation in the sheltered location (CC), while the CH, showed the lowest value of annual defoliation (Table 4).

Table 4. Mean production ($cm^2 \cdot shoot^{-1} \cdot d^{-1}$) and mean defoliation (i.e. including all consumers, $cm^2 \cdot shoot^{-1} \cdot d^{-1}$) of *C. nodosa* meadows at each sampling site. Data only consider shoots from their original site. Data are showed as mean ± s.e.

	Mean Production ($cm^2 \cdot shoot^{-1} \cdot d^{-1}$)	**Mean Defoliation ($cm^2 \cdot shoot^{-1} \cdot d^{-1}$)**	**% Defoliation**
CC	0.818 ± 0.034	0.606 ± 0.33	74.08
ST	0.893 ± 0.042	0.603 ± 0.34	67.52
BC	0.676 ± 0.18	0.389 ± 0.256	57.54
CH	0.616 ± 0.12	0.287 ± 0.261	46.59

Table 5. Statistical results of the Kruskall Wallis test examining the mean production ($cm^2 \cdot shoot^{-1} \cdot d^{-1}$) and mean defoliation rates ($cm^2 \cdot shoot^{-1} \cdot d^{-1}$) values at each sampling site. Bold font indicates significance differences ($p<0.05$).

	df	**X^2**	***p*-value**
Production			
Season	3	33.905	**< 0.001**
Location	4	98.789	**< 0.001**
Season:Location	19	151.24	**< 0.001**
Defoliation			
Season	4	38.421	**< 0.001**
Location	3	19.554	**< 0.001**
Season:Location	19	67.448	**< 0.001**

On the other hand, considering all uprooted shoots in each location, the size effect revealed a decrease in the defoliation rate for leaves transplanted into CC in summer (Figure 5

and 6). Therefore, we found higher defoliation rates in the CC native leaves compared to the rest of transplanted leaves. Leaves from CH also received lower defoliation rates in the three locations transplanted (i.e. CC, ST and BC) in spring. A null defoliation was found in winter in BC and CH. In addition, leaves from BC and CH were also not consumed in autumn and winter for all other locations (Figures 5 and 6).

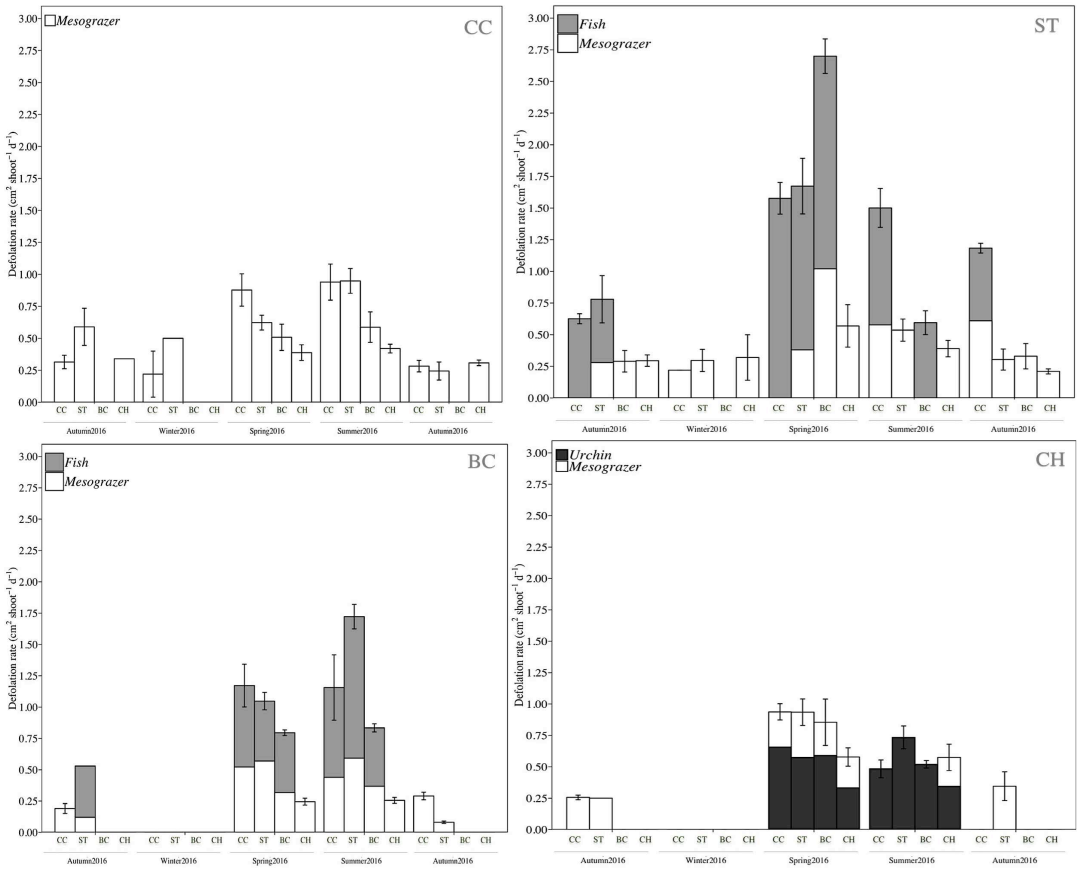

Figure 5. Spatial-temporal differences in defoliation rates (cm^2 $shoot^{-1}$ d^{-1}) by consumers (mesograzers, fish and urchins) of *C. nodosa* populations from the four experimental locations (from low to high exposure, CC: Caño Cortadura; ST: Santibáñez; BC: Bajo de la Cabezuela; CH: El Chato). Data are represented as mean ± s.e. (n=10).

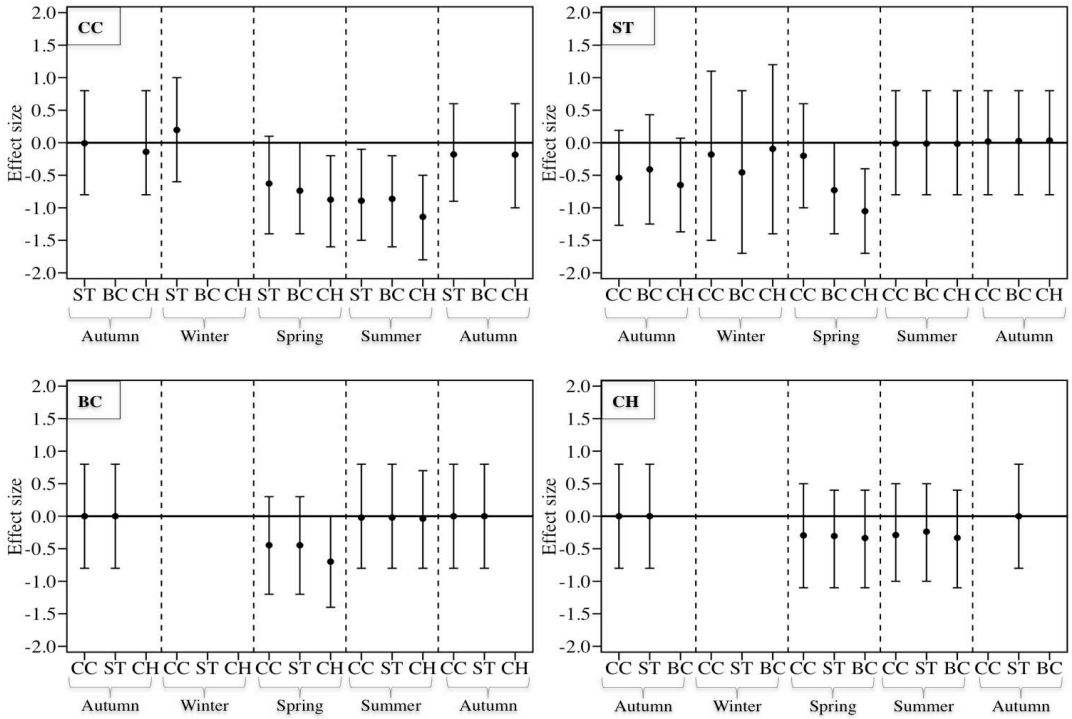

Figure 6. Effect sizes (n=10) of *C. nodosa* defoliation in each sampling site for transplanted shoots from respect to the native shoots. From low to high exposure, CC: Caño Cortadura; ST: Santibáñez; BC: Bajo de la Cabezuela; CH: El Chato.

Discussion

Effects of hydrodynamics on Cymodocea nodosa palatability

Leaf traits differed between locations and seasons. This result is agreement with De los Santos (2013) who observed intra-specific variability in C. nodosa plants inhabiting areas with different hydrodynamic conditions. However, our study goes further, and did not only showed the C. nodosa plasticity as a key element for the mechanical performance of these plants (De los Santos et al. 2013), but also clearly demonstrated that this acclimation altered their value as food source (palatability). Overall, the exposure to different hydrodynamic conditions seemed to affect some leaf traits of C. nodosa leaves, since plants from the locations subjected to high exposure to waves (CH) and moderate exposure to currents (BC), presented higher structural traits (i.e. high NDF, %C and thickness), lower nutritional quality

(low %N), and thinner and shorter leaves conferring low palatability to these tissues, as indicated the low defoliation rates measured (Figure 5). Meanwhile, plants from CC and ST (low hydrodynamics exposure) had higher nutritional quality (higher %N), lower strength (i.e., low FTA and FTS) and lower structural traits (i.e. low NDF, %C and thickness), which can render more palatable tissues to consumers (Heck and Valentine 2006; Cebrián et al. 2009) as also indicated by the higher defoliation rates measured (Figure 5).

Hydrodynamics influence on Cymodocea nodosa defoliation

The results evidence that environmental conditions of the different locations influence leaf traits and consequently, the defoliation rates of *C. nodosa*. A substantial defoliation rate of *C. nodosa* was recorded in the examined meadows, reaching a gross annual estimate between ca. 46%-74% of the annual leaf production (Table 3). The highest rate of mean defoliation was found at the sheltered location (i.e. CC), where leaves rendering more palatable, following a downward pattern towards the most exposed locations. It is well documented that some seagrass herbivores are selectively fed of seagrass leaves (Cebrián and Lartigue 2004; Heck and Valentine 2006; Prado and Heck 2011), being the nutritional quality (McGlathery 1995; Cebrián and Lartigue 2004; Goecker et al. 2005) as well as structural and chemical deterrents (Vergés et al. 2007a; b) a key elements to define leaf palalability and then, to determine the feeding decisions. In general, those leaves adapted to higher hydrodynamic conditions (BC and CH), rendering the less palatability, obtained lower rates of defoliation at the sampling sites, which is mainly observed in leaves coming from CH, even during the months of spring and summer corresponding to the greater abundances of consumers. Hence, de demonstrate how hydrodynamics modulate the seagrass palatability by varying leaf properties due to their environmental acclimatization and consequently, those plants living in less exposed places should have more palatable leaves. In addition, the higher palatability leads to greater defoliation by the herbivores, since more palatable leaves may be more consumed.

On the other hand, hydrodynamics not only influences on leaf properties, but also over their consumers. In previous works it has been observed how the physical stress caused by currents on mesograzers inhabiting in seagras communities negatively influenced their feeding and foraging capacity (Schanz et al. 2002; Jiménez-Ramos et al. 2017). Based on this statement, in addition to finding a negative pattern between hydrodynamics exposure and leaf defoliation (ie, higher exposure, less leaf defoliation), we found that the leaf selection by consumers took place in the less exposure sites (CC) where consumers had lowest physical stress (Figure 5).

It was in CC where the size effect revealed a differential defoliation rates between transplanted leaves vs native leaves, being the native more palatable, and therefore those that obtained higher rates of defoliation.

Feeding activity of grazers

Mesograzers (including isopods, decapods, amphipods) accounted for the remaining 40.28% of total annual losses to herbivores, obtaining the highest rate defoliation in CC and the lowest values in BC. These spatial differences may arise from variability in local hydrodynamic conditions, since shallow and sheltered sites seem to sustain greater feeding rates than those unsheltered (Schanz et al. 2002). Jiménez-Ramos et al. (submitted) demonstrated that habitat complexity on seagrass meadows (i.e. shoot density), had effects on mesograzers feeding, keeping higher feeding rates at high density under low and moderate flows. In our case, the higher defoliation rates of *C. nodosa* meadows were related to populations with high shoot density, and in areas exposed to low hydrodynamic conditions (i.e. CC and ST). In addition, in areas subjected to high hydrodynamics (BC and CH), the highest rates of annual defoliation occurred when high shoot densities (spring and summer) were also recorded in the populations, but it also was coincident with a higher abundance of mesograzers.

On the other hand, the fish defoliation (probably by *Salpa sarpa*) was showed only for ST and BC locations. Indeed, fishes were consistently responsible for the majority of the removed leaf material at these locations and during spring and summer, confirming the preeminence of fish in *C. nodosa* and being similar for *Posidonia oceanica* herbivory (Cebrián et al. 1996; Tomas et al. 2005a). Defoliation by fishes clearly differed among sampling times (contribution to total seagrass leaf losses ranged from 143% in spring to 62% in autumn). These differences are most probably caused by the seasonal migratory and feeding behaviour of this fish species, especially of the main consumer *S. sarpa* (Verlaque 1990; Tomas et al. 2005a). However, spatial differences in the timing of obtained defoliation rates can be, at least in part, attributed to spatial differences in the periods of arrival and departure of fishes in spring and autumn, respectively. It is remarkable to note that defoliation values for fishes may be slightly overestimated due to the fact that lateral bites exerted by large individuals may enhance leaf break by the effect of wave action; however, these cases were rare (two samples of BC leaves placed in BC during Spring). Meanwhile, sea urchins accounted for the remaining 90% of total annual losses to herbivores in the CH. Defoliation rates by sea urchins also differed among sampling times, being null in autumn and winter. These differences can be attributed to the influence of seasonal

variables on feeding activity such as temperature and the nature and abundance of trophic resources (Régis 1979; Frantzis and Gremare 1993).

Ecological implications

Numerous attempts have been made to meet the tremendous seagrass losses the marine environment has faced worldwide. Artificial transplanting of shoots and spreading of seeds from intact meadows to non-vegetated coastal sediment are the most applied techniques (Calumpong and Fonseca 2001). This work has showed how the seagrass acclimation at each location determines the defoliation rates directly (i.e. by hydrodynamics effects over consumers) and indirectly (i.e. leaf traits changes). Therefore, the plant transplantation from one place to another could generate implications in the destination of the production in each location. For example, if plants from the CC, which obtained the highest percentage of defoliation, were transplanted in the CH, we would have an increase in defoliation in this site, modifying the amount of primary production of *C. nodosa* transferred to the system. Therefore, transplanting to sites in which environmental conditions approach those of the donor site as much as possible is the most obvious way to improve the survival chances of the transplants. Hence, our findings support the statements that environmental conditions and biotic factors (i.e. species of consumers) should be keys requirements to be considered in the management of seagrass restoration by their influence on the structure and dynamics of seagrass communities.

Conclusions

In summary, given the local distribution of consumers and the direct rates of leaf defoliation reported in the present study, evidence is sufficient to show that local environmental conditions influences on *C. nodosa* palatability (i.e. varying intra-specific traits) and consequently, on the defoliation rates of seagrass leaves. Moreover, we highlight the influence of physical stress over consumers, which could modulate their feeding behaviour. Our results suggest that the grazers feeding closely control the productivity of seagrass meadows from low exposure sites, whereas herbivory pressure in high hydrodynamics exposure meadows may have lower rates. In addition, we highlight the influence of leaf type transplantation from one place to another on the destination of productivity mediated by herbivores.

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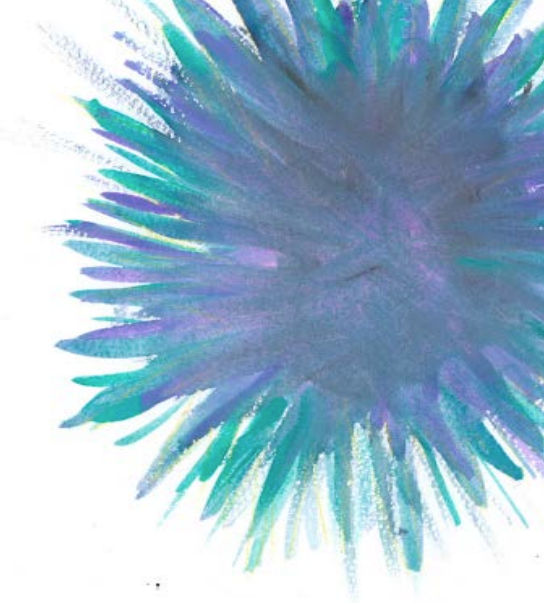

CHAPTER 3

Food choice effects on herbivory: intra-specific seagrass palatability and inter-specific macrophyte palatability in seagrass communities

Estuarine, Coastal and Shelf Science, submitted

Ninguna acción buena se pierde en este mundo. En algún lugar quedará para siempre.
No good action is lost in this world. Somewhere will be forever.

Vicente Ferrer

Food choice effects on herbivory: intra-specific seagrass palatability and inter-specific macrophyte palatability in seagrass communities

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Abstract

Interactions between the palatability and abundance of different food sources may influence herbivory patterns in seagrass-dominated communities. In addition, intra-specific differences in nutrient and structural quality of leaves may also alter seagrass palatability and generate different rates of consumption within these communities. We offered two temperate seagrasses species and two other macrophytes, both of which occur at the same location as seagrasses but represent the extremes of palatability, to a generalist herbivore *Paracentrotus lividus* (purple sea urchin). Using feeding assays, we compared the consumption rates in individual (single plant species) and combined diets at different food availabilities. Intra-specific differences between seagrass species growing at different locations (inner and outer bay) were indeed found to significantly modify the consumption rate for one species. Structural traits such as carbon content were linked to the low consumption found in *Cymodocea nodosa* from the inner bay location. In addition, we found that the co-occurrence of different macrophyte species can result in preferential consumption of the more palatable macrophyte with high nutritional content and low structural defence over seagrasses, especially when *P. lividus* has an abundant food supply. Overall, our findings suggest that intra- and inter-specific differences in seagrass traits and the relative abundance and spatial distribution of other macrophytes may explain the variability in patterns of herbivory found within seagrass communities

Introduction

Herbivory is a key factor in the structure and distribution patterns of seagrass communities, influencing the transfer of energy and matter through the whole ecosystem (Poore et al., 2012). Seagrasses co-occur with other seagrass and macrophyte species (e.g. seaweeds, epiphytes, etc.) creating mixed communities, and these co-occurring species may represent additional food sources for herbivores (Hulme, 1996). Over the last 10–15 years, there have been a growing number of studies on the importance of macrophyte palatability on herbivory rates. These studies show that herbivores can shift into different patterns of vegetation consumption when exposed to a mixture of macrophytes with different degrees of palatability, which may lead to consequences at both the community and ecosystem levels (Valentine and Heck, 1999; Heck and Valentine, 2006; Del Río et al., 2016). These effects of herbivory may even match the magnitude of changes in community diversity (species richness and abundance) brought about spatial and seasonal variation (Fourqurean et al., 2001; Gullström et al., 2006). Thus, determining the macrophyte traits that underlie differences in palatability and thus the differential feeding behaviour of herbivores may be crucial in forecasting the response of coastal communities to a changing world.

Previous studies on the preferences of herbivores feeding on seagrasses and other macrophytes have reported that nutritional, structural and chemical traits may determine leaf palatability and thus regulate their attractiveness as a food source as well as subsequent consumption rates (Cebrian et al., 2009; Mariani and Alcoverro, 1999; Prado and Heck, 2011; Zapata and McMillan, 1979). Nutritional quality (e.g. nitrogen content) has been considered to be the main factor regulating feeding decisions by consumers in terrestrial and aquatic ecosystems (Cebrian et al., 2009; Elser et al., 2000). However, experimental evidence also suggests that the structural traits of seagrass leaves, related to leaf mechanical resistance, may also play a key role in regulating plant-herbivore interactions in seagrass ecosystems (Prado and Heck, 2011; Vergés et al., 2011).

Inter-specific differences in palatability are expected since seagrass species bear different C:N ratios (Duarte, 1992), fibre content (de los Santos et al., 2016; Mariani and Alcoverro, 1999) and natural product concentrations (Grignon-Dubois et al., 2012; Vergés et al., 2007a; Jiménez-Ramos et al., 2017). While there are some studies focusing on inter-specific differences in seagrass palatability and their influence on feeding decisions by consumers (Martínez-Crego et al., 2016; Tomas et al., 2011), little information is available regarding intra-specific differences (i.e. differences within a single species). These intra-specific differences can result from either genotypic or phenotypic variability (Koricheva, 2002). The contribution of these mechanisms in determining palatability will depend on the specific morphological response to environmental conditions since, for instance, internal nitrogen content may be

altered by nutrient enrichment (Brun et al., 2002; Tomas et al., 2011; Villazán et al., 2013), carbohydrate reserves may be altered by light levels (Brun et al., 2002 and 2008), the abundance of phenolic compounds may be reduced by high CO_2 levels (Arnold et al., 2012; Jiménez-Ramos et al., 2017) and fibre content may be altered by hydrodynamic conditions (de los Santos et al., 2013).

The seagrasses *Cymodocea nodosa* and *Zostera noltei* are found at several locations in Cádiz Bay (southern Spain) with different degrees of exposure to waves and currents, showing contrasting morphologies among locations (Brun et al. 2003a; Brun et al. 2005; de los Santos et al., 2013). Moreover, these seagrasses co-occur with other macrophytes that have contrasting levels of palatability, such as *Ulva* sp. or *Spartina maritima*, with the latter found often as cast material within seagrass beds. In this study, these two temperate seagrass species (from two different locations) and two other macrophytes thriving at the same locations, representing extremes in the range of palatability, were offered to the generalist herbivore *Paracentrotus lividus* (purple sea urchin). Herbivore feeding choice and behaviour were compared in feeding assays using individual and combined diets at different food availabilities (i.e. biomass quantity of each species). In this work, we aim to answer to the following questions: 1) whether intra-specific differences modify leaf properties in two seagrass species, and thus determine palatability and subsequent consumption rates by herbivores; 2) whether food source diversity results in differential feeding rates by herbivores; and 3) whether the abundance of food generates a differential pattern of consumption by herbivores.

Material and Methods

Collection of plant samples

The seagrasses *Cymodocea nodosa* (Ascherson) and *Zostera noltei* (Hornemann) were sampled in two locations of Cádiz bay (SW Spain) at the end of July: the inner and the outer bay (Figure 1).

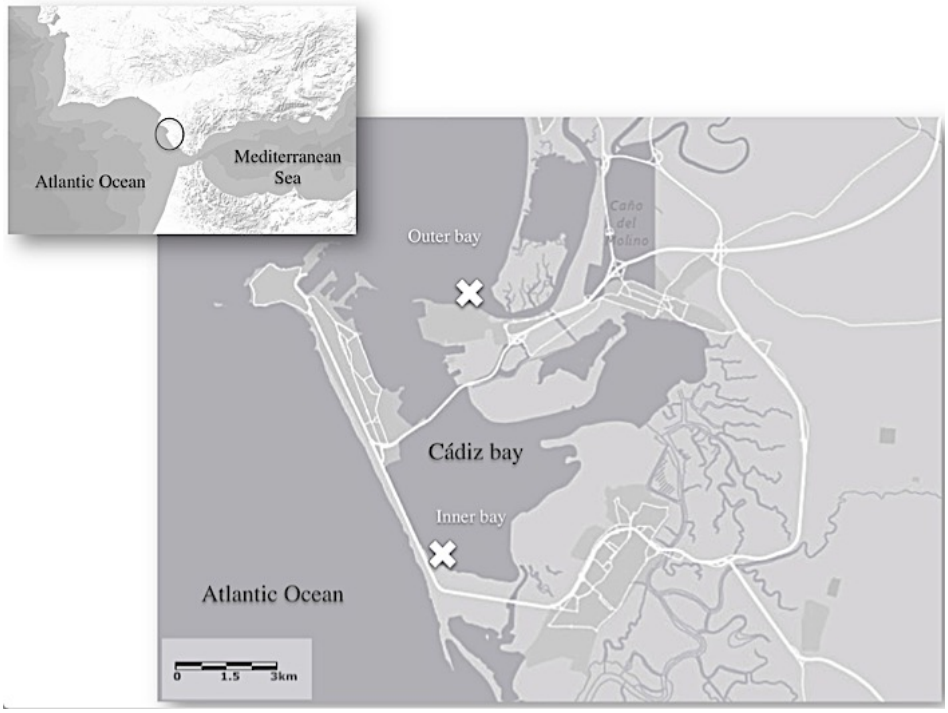

Figure 1. Map of the study area in SW Spain showing the two locations (cross mark) of *Cymodocea nodosa* and *Zostera noltei* beds.

These areas differ mainly in hydrodynamic forces and depth. Located in the inner bay, Santibáñez (36º1'N; 06º15'W) is protected from the action of large waves but is strongly influenced by semidiurnal tides with a mean amplitude of 1.5 m (Alvarez et al., 1999). The mean velocity ranges approximately from 0.015 to 0.08 m $\cdot s^{-1}$ during a tidal cycle (Morris et al., 2013). Located in the outer bay, El Bajo de la Cabezuela (36º31'N; 06º14'W) is a shallow inlet. This area is characterized by semidiurnal mesotides with relatively higher mean velocities ranging from 0.05 to 0.25 m $\cdot s^{-1}$ (Brun et al., 2009). Both these areas are covered by patches of *Z. noltei* in the intertidal zone and by *C. nodosa* in the shallow subtidal zones and some areas in the intertidal zone (Brun et al., 2005; F. G. Brun et al., 2003). Care was taken to collect shoots with intact vertical rhizomes to minimize chemical changes from occurring over time in the detached plants. Specimens of *Ulva* sp. and *Spartina maritima* were collected from the same area at the same time as the seagrasses. *Ulva* sp. was selected because of its high nutritional content and palatability, and was expected to be the most consumed food. *Spartina maritima*, in contrast, was considered as a non-consumption control, because of its rigid structural properties. In order to define palatability, we measured the morphological (i.e. length, width

and thickness), nutritional (i.e. nitrogen and organic matter content) and structural properties (i.e. carbon and fiber content) of the leaves. Prior to any measurement, macrophyte specimens were carefully selected for healthy appearance (without necrosis or bites). All seagrasses and *S. maritima* plants were kept in a 24-litre tank filled with aerated natural seawater at room temperature (20ºC) until the beginning of the experiment. *Ulva* sp. was immediately frozen at -20ºC to prevent *Ulva* sp. growth during the assays. Seagrass leaves of each species were carefully cleaned of epiphytes with soft laboratory paper in order to avoid damaging leaf surfaces.

Collection of sea urchins

Paracentrotus lividus (purple sea urchins) were collected from a nearby rocky shore, La Caleta, in outer Cádiz Bay (SW Spain, 36º31'39"N; 6º18'46"W). This area was chosen because the most stable population of *P. lividus* is found here. After receiving permission from the local environmental agencies, *P. lividus* individuals were collected at a depth of 2 meters. Harvesting was carefully carried out by snorkelling, avoiding damage to the organisms. *P. lividus* size varied between 5-7 cm in diameter (average adult size was 6.3 ± 0.72). The collected individuals were kept in coolers with seawater and transported to the laboratory within one hour. Upon arrival, *P. lividus* were placed in aerated tanks (4 tanks of 30 liters each, 36 individuals in each tank) and were fed with the macroalgae *Ulva* sp. for 3 days during the acclimation period until the beginning of the experiment.

Morphological analyses

Ten healthy experimental plant units (EPUs) for each seagrass species (i.e. no necrotic, bitten or broken leaves) from the initial pool of plants were selected from each location (i.e. *C. nodosa* and *Z. noltei* from the outer and inner bays). An EPU consists of one vertical shoot with its first rhizome segment (i.e. first internode). We measured total leaf length, width and thickness (measured 2 cm above the ligule) in all the selected EPUs with a digital calliper (Mitutoyo 500 AOS) and a thickness gauge (Mitutoyo 7301). Blade cross-sectional area (CA, mm^2) was calculated assuming a rectangular shape, and leaf volume (V, mm^3) using the total length and CA. Leaf thickness was also measured in the initial pool of *Ulva* sp. and *S. maritima* samples (n=10).

Nutritional and structural analyses

Nutritional and structural measurements were examined in 10 EPUs from each of the two seagrass species collected from each location *(*i.e. *C. nodosa* and *Z. noltei* from inner and outer bay*)* and also in the initial pool and *Ulva* sp. and *S. maritima*. Total C and N content in

tissue were measured using a Perkin-Elmer 2400 elemental analyser. Nutrient content was expressed in molar ratios, and the final results were based on dry biomass.

The dry biomass of each sample was used to determinate fibre content using the method from Van Soest et al. (1991), further modified by de los Santos et al. (2012). Namely, approximately 30 mg of dry biomass was boiled in 2 ml of neutral detergent for 1h. These samples were subsequently centrifuged (5 min at 2,500 g), and the resulting pellets were washed with distilled water (x2), ethanol (x2) and acetone (x1), with a centrifugation step (as described above) following each washing step. The final pellet, which was free of non-cell-wall components and chlorophyll, was dried overnight in an oven (60 ºC) and subsequently weighed. The neutral detergent fiber content (NDF) of the macrophyte tissues was obtained from the difference in mass before and after the procedure, and expressed as a percentage of dry biomass.

The water content in the samples was calculated as the weight difference between fresh and dried biomass after 72 hours in the oven (60ºC). Total organic matter was determined by combustion of dry samples at 500ºC for 5 h. The results are expressed on the basis of ash-free dry weight.

Experimental set up

To experimentally examine whether intra and inter-specific differences in seagrass tissues, and diversity (number of species and abundance) of food sources affect the feeding preference of the purple sea urchin, we conducted a suite of no choice and food-choice feeding assays, also using *Ulva* sp. and *S. maritima*.

The experiment consisted of 3 different feeding assays: i) individual diet (ID, no choice assays), ii) combined diet with high food availability (CDHA, food choice assays), and iii) combined diet with low food availability (CDLA, food choice assays).

i) Individual diet assays (ID)

A total of six individual feeding assays (see below: U, CnO, CnI, ZnO, ZnI, SP) were carried out for each food choice group as independent trials. For each replicate (n=6), three independent *P. lividus*, collected randomly from the pre-acclimation tanks and starved for 24 h prior to the feeding assay, were fed with 6 g fresh weight (WW) of each food group separately: *Ulva sp.* (U), *C. nodosa* from outer (CnO) and inner (CnI) bay, *Z. noltei* from outer (ZnO) and inner (ZnI) bay, and *Spartina maritima* (SP). Since both seagrass species (*C. nodosa* and *Z. noltei*) had been collected from two different locations (inner or outer area of Cádiz bay) to explore the intra-specific differences in leaf traits, assays within species from these locations were

considered as independent assays. Rocks served as supports for plants in the tanks and as an optimal substrate for sea urchins.

ii) Combined diet assays (CD)

Two types of combined diet assays were performed. One treatment, namely high food availability (CDHA), was carried out with 6 g WW for each food group for a total of 24 g WW. The other treatment, namely low food availability (CDLA), contained 1.5 g WW of each food group for a total of 6 g WW. Again, seagrass specimens were divided between locations (i.e. inner and outer bay were assayed separately). All food groups were maintained under the same ratio of food to *P. lividus* density to maintain the same probability of *P. lividus* encountering food.

All the feeding assays (U, CnO, CnI, ZnO, ZnI, SP, CDHA and CDLA) were conducted over six consecutive days (8 different assays with 6 replicates). Every day was considered as an independent replicate for each treatment (6 replicates for each feeding assay). All the feeding assays were run with three different individuals of *P. lividus* (different individuals in each treatment and replicate), which had been randomly collected from the pre-acclimation tanks and starved for 24 h prior to the feeding assay. Each feeding assay was run in a 24-litre tank for 24 h under constant temperature at 20ºC. After this period, the remaining plant biomass was collected from tanks, weighed and dried. In all cases, the photoperiod consisted of 8h light and 16h of darkness because *P. lividus* usually exhibit nocturnal activity (Bouderesque *et al*., 2001). Simultaneously, autogenic controls were performed to account for potential changes not due to grazing (i.e., fresh biomass of *Ulva* sp. and seagrass not exposed to *P. lividus*). Results showed no significant autogenic changes in *Ulva* sp. and seagrasses during the control tests. Regardless, the consumption rates were corrected using the autogenic controls from each assay.

At the end of each assay, *P. lividus* were weighed to control for their size (average of 254.89 ± 25.43 gWW) and each macrophyte was weighed for their final biomass. Consumption rates were expressed as biomass consumption per *P. lividus* individual per day.

Statistical analyses

S. maritima was not consumed in any of the assays and was not further considered in the statistical analyses. A one-way ANOVA and Tukey's post hoc analyses were applied to assess significant differences in the no-choice feeding assays (individual diet, ID). Differences in consumption rate from the combined diets (CDHA, CDLA) were analysed following Prince et al. (2004), using Hotelling's multivariate (T^2) test. For intra-specific differences in seagrass traits, we analysed differences between plants collected from the inner and outer bay

using unpaired t-tests. One-way ANOVA and Tuckey's post hoc analyses were applied to the whole tissue traits of all the species assayed (thickness, fibre content, C and N content, C/N ratio and OM content). Data were transformed (i.e. consumption rates) when necessary to meet ANOVA assumptions of normality (Shapiro-Wilks test) and homogeneity of variances (Barlett test). Moreover, when ANOVA assumptions were not satisfied (i.e. fibre content and thickness), a non-parametric comparison (Kruskal Wallis matched pairs test) was applied to assess statistically significant differences. The significance level was set at 0.05. To assess relationships between tissue traits, a principal component analysis (PCA) was run using all the macrophytes assayed (*C. nodosa* and *Z. noltei* from the inner and outer bay, *Ulva* sp. and *S. maritima*). PCA was run with scores from the correlation matrix, in which the variable scores reflect the correlation to the components and the angles between variables are proportional to their covariances. The significance of the PCA results was further confirmed by Pearson correlations between each variable. Statistical analyses were computed with R statistical software 3.0.2 (R Development Core Team 2013).

Results

Leaf characteristics: comparison across species

The seagrass species were significantly different in morphological, nutritional and structural traits (Figure 2, Table 1). In addition, intra-specific differences (i.e. inner and outer bay) were also found in both species. CnI exhibited significantly higher C content (higher C:N ratios) and thinner leaves. Regarding *Z. noltei*, ZnI showed significantly lower N content, as well as higher C:N ratios and thinner leaves (Figure 2).

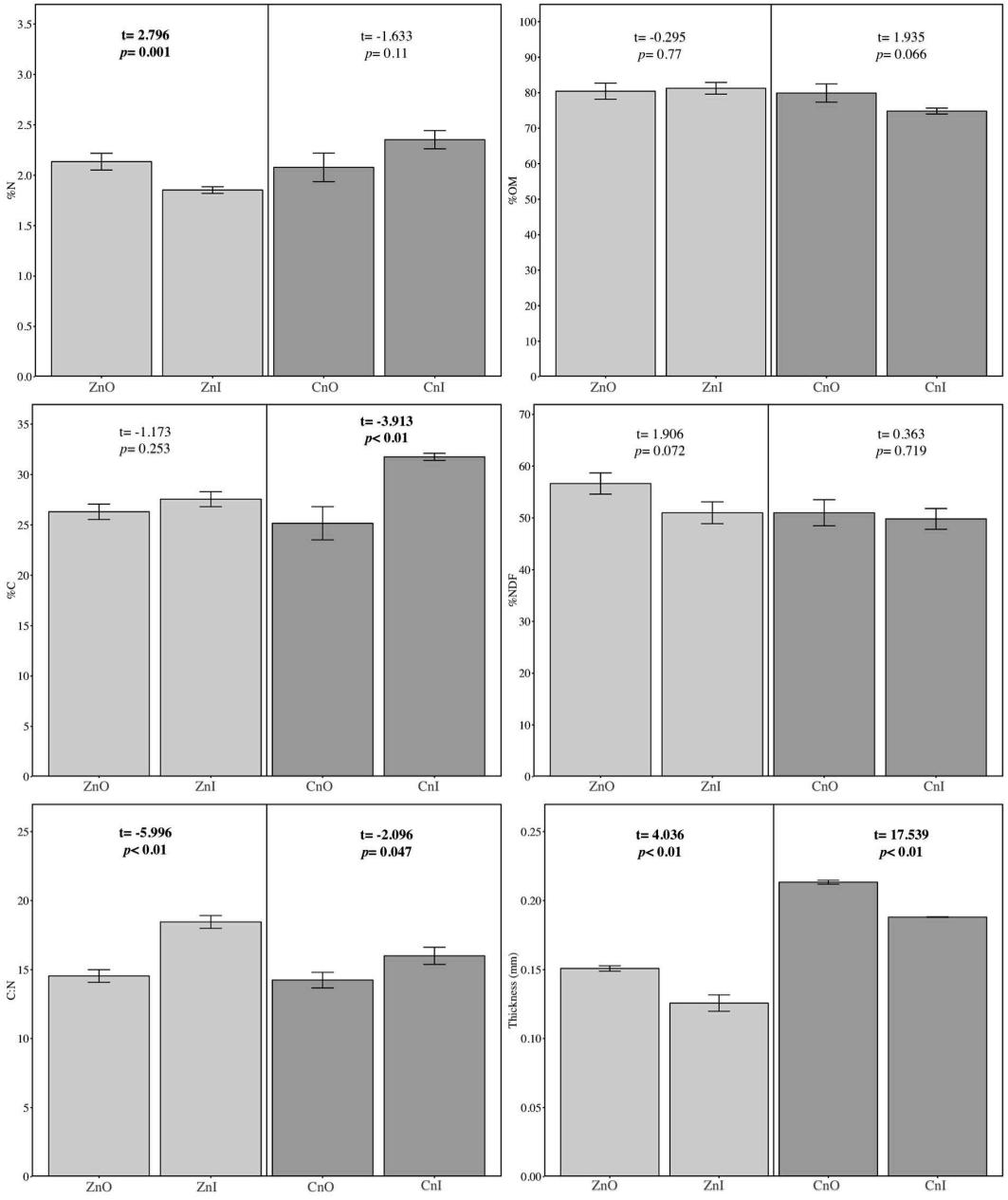

Figure 2. Leaf traits of *Cymodocea nodosa* and *Zostera noltei* plants from inner and outer bay. Data are expressed as mean ± s.e (n=10). For each leaf trait, statistics from an unpaired t test and sample sizes (inside bars) are shown. Bold letters show significant differences (p<0.05).

Table 1. Leaf properties for the whole set of macrophytes assayed. C: leaf carbon; N: leaf nitrogen: C:N atomic ratio; NDF: leaf fiber content; OM: leaf organic matter; Th: leaf thickness. Data are expressed as mean ± s.e. (n=10). ZnO: *Zostera noltei* from outer bay; ZnI: *Zostera noltei* from inner bay; CnO: *Cymodocea nodosa* from outer bay; CnI: *Cymodocea nodosa* from inner bay. Different superscript letters indicate significant differences in leaf properties among species (p value < 0.05) from one-way Anova analyses.

	%C	%N	C:N	%NDF	%OM	Th (mm)
***Ulva* sp.**	24 ± 1.1^{a}	2.7 ± 0.1^{a}	11 ± 0.7^{a}	49 ± 2.7^{a}	87 ± 1.2^{a}	0.09 ± 0.003^{a}
ZnO	26 ± 0.7^{a}	2.1 ± 0.1^{bc}	14 ± 0.5^{b}	57 ± 2.9^{b}	80.5 ± 2.8^{ab}	0.15 ± 0.01^{b}
ZnI	28 ± 0.8^{a}	1.8 ± 0.05^{c}	18 ± 0.6^{c}	48 ± 2.3^{a}	81 ± 2.1^{a}	0.12 ± 0.01^{a}
CnO	25 ± 1.9^{a}	2 ± 0.2^{bc}	14 ± 0.7^{b}	51 ± 2.4^{a}	80 ± 3^{ab}	0.2 ± 0.01^{b}
CnI	32 ± 0.3^{b}	2.3 ± 0.1^{ab}	16 ± 0.8^{bc}	50 ± 2.9^{a}	75 ± 0.9^{bc}	0.18 ± 0.002^{b}
Spartina maritima	39 ± 0.7^{c}	2.1 ± 0.1^{bc}	22 ± 1.1^{d}	62 ± 2.1^{b}	73 ± 0.5^{c}	0.3 ± 0.02^{c}

When considering all the macrophytes assayed, the one-way ANOVA test showed significant differences in tissue traits, which indicate differences in palatability attributes among food groups (Table 1). *Ulva* sp. showed the highest N and OM content but the lowest C content, NDF and thickness in comparison to the other species. In contrast, *S. maritima* displayed the opposite pattern (Table 1). Significant negative correlations between C content and OM content, and between OM content and leaf thickness were found (Table 2). NDF content was also negatively correlated with OM and positively correlated with the above-mentioned structural traits.

Table 2. Pairwise Pearson's correlation coefficient among leaf properties. Correlations were performed between averaged values for each species (n=10). The corresponding statistical significance is given by asterisks (*** P<0.001. ** P< 0.01. * P<0.05). C: leaf carbon; N: leaf nitrogen; C:N ratio; NDF: leaf fiber content; OM: leaf organic matter; Th: leaf thickness; CR: Consumption rate.

	%C	%N	C:N	%NDF	%OM	Th (mm)	CR (gWW ind^{-1} d^{-1})
%C	-	-0.26	0.94**	0.37	-0.81*	0.77	-0.89*
%N		-	-0.49	0.03	0.09	-0.14	0.6
C:N			-	0.26	-0.64	0.6	-0.94**
%NDF				-	-0.75	0.71	-0.2
%OM					-	-0.99***	0.64
Th (mm)						-	-0.66
CR (gWW ind^{-1} d^{-1})							-

Differences in tissue traits clearly discriminate *Ulva* sp., seagrasses and *S. maritima* along component I of the PCA, which explained 71.2% of the variance (Figure 3). N content, which was not correlated to any other trait, was the only variable that was significantly correlated with component II of the PCA, which explained 17.9% of the variance.

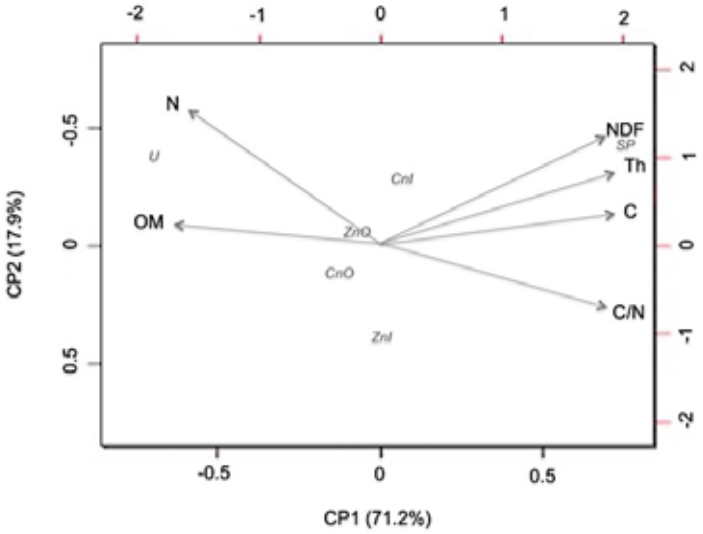

Figure 3. Relationships between tissues traits of the whole set of macrophytes assayed. U: *Ulva* sp.; ZnO: *Zostera noltei* from outer bay; ZnI: *Zostera noltei* from inner bay; CnO: *Cymodocea nodosa* from outer bay; CnI: *Cymodocea nodosa* from inner bay; SP: *Spartina maritima*. Trait loadings (grey lines) reflect the correlation to the components and the angles between lines are proportional to their co-variances.

Consumption rates of macrophytes

In the individual diet assays (ID), *P. lividus* showed differential consumption rates for food groups (Figure 4). No significant differences were recorded between consumption rates of *Ulva* sp. and the two seagrass species, with the exception of CnI, which was less consumed (df= 5, F= 9.936, $p < 0.01$). In the choice assays (combined diet), however, there were differences in the consumption rates between *Ulva* sp. and seagrasses from both areas, depending on food availably (high *vs.* low) (Figure 5).

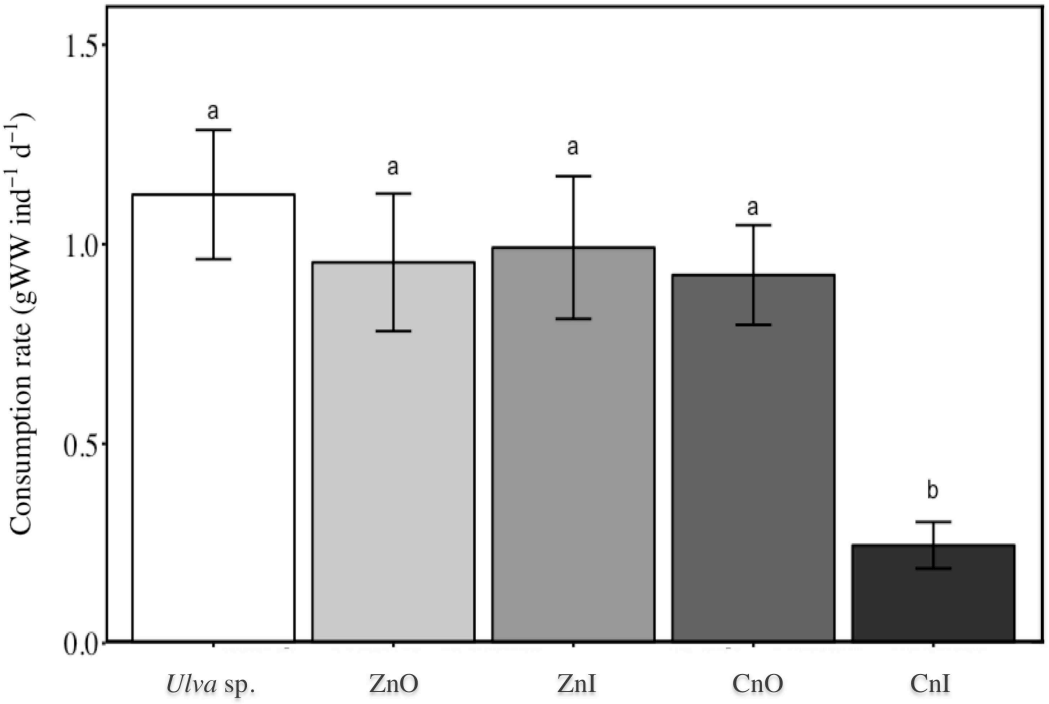

Figure 4. Consumption rates of macrophyte tissues in individual diet (ID) assays (g wet weight [WW] $\cdot$ind^{-1} $\cdot$ d^{-1}; mean $\pm$ s.e.) by the sea urchin *Paracentrotus lividus* for the different species assayed. ZnO: *Zostera noltei* from outer bay; ZnI: *Zostera noltei* from inner bay; CnO: *Cymodocea nodosa* from outer bay; CnI: *Cymodocea nodosa* from inner bay. Different letters indicate significant differentes among consumption rates at $p<0.05$ (Anova 1-way; $n=6$).

The chlorophyte *Ulva* sp. was consumed at higher consumption rates than seagrass species under high food availability (CDHA) (p = 0.02 in Figure 5A; p = 0.019 in Figure 5C), but there were no significant differences at low food availability (CDLA) (Figure 5B & D). Moreover, the consumption rate for different food groups under CDHA was similar those found in the ID assays (Figure 5A, C and 6A). However, the sum of plant material consumed under CDHA was much higher than under ID, while this sum was similar under CDLA. In all cases, food sources were not depleted during the experiments.

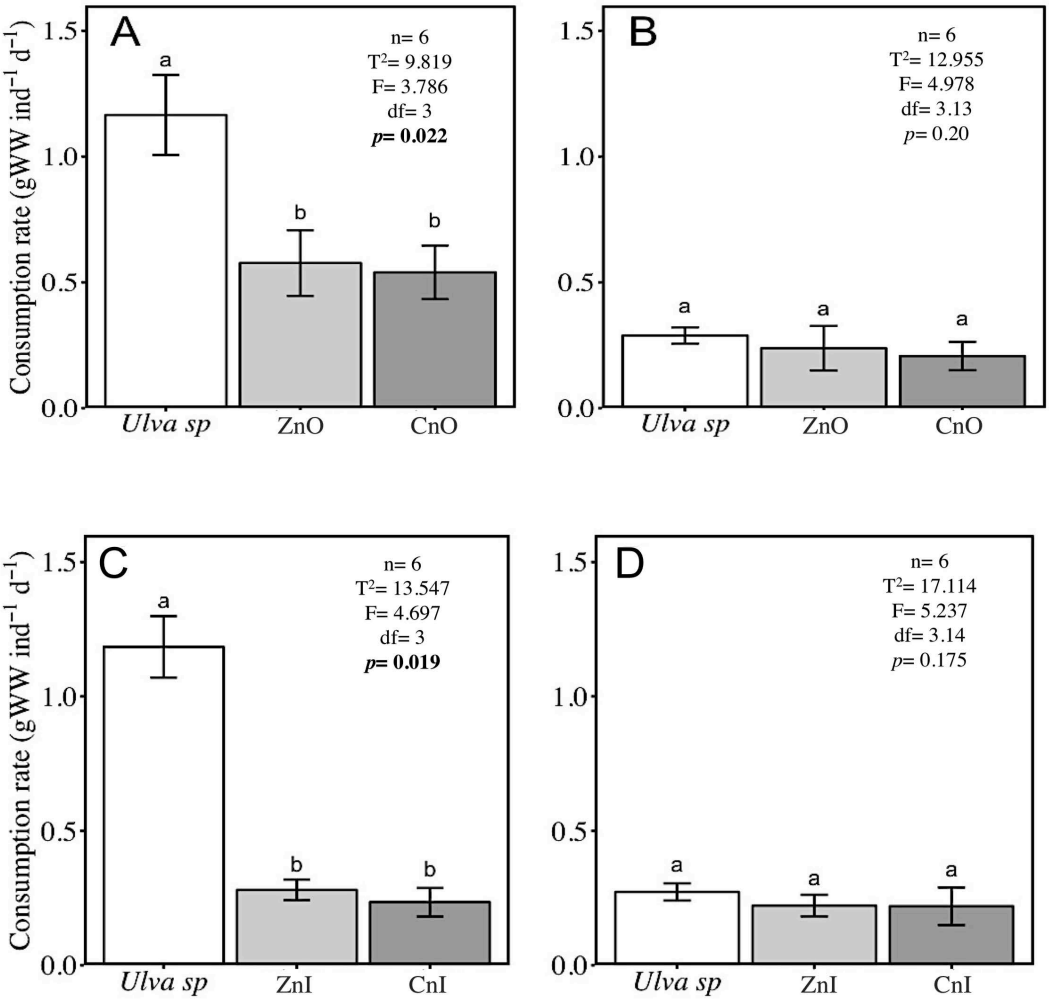

Figure 5. Consumption rates of seagrasses and *Ulva* sp. in combined diet assays (g wet weight [WW] $\cdot ind^{-1} \cdot d^{-1}$; mean ± s.e.) by the sea urchin *Paracentrotus lividus*. ZnO: *Zostera noltei* from outer bay; ZnI: *Zostera noltei* from inner bay; CnO: *Cymodocea nodosa* from outer bay; CnI: *Cymodocea nodosa* from inner bay. A-C. High food availability, CDHA (6 g WW/species). B-D. Low food availability, CDLA (1.5 g WW/species). Different letters indicate significant differences among groups at $p < 0.05$ (Hotelling's test; n=6).

Discussion

The results show that differences in traits related to palatability (morphological, nutritional and structural) of the assayed food groups, as well as the diversity of food sources (number of species and abundance), determined the probability of being consumed. The two seagrass species displayed differences in morphological, nutritional and structural traits, at both the intra- and inter-specific levels, according to the different abiotic and biotic conditions of their habitats (i.e. inner *versus* outer bay, Figure 1). For instance, while CnO and ZnO showed higher leaf thickness, they also possessed lower C:N ratios than those from the inner bay. However, these differences were only translated into significant differences in seagrass consumption for *C. nodosa*, probably due to the large intra-specific structural differences in this species as observed by past studies (i.e. Marinai and Alcoverro, 1999; Prado and Heck, 2011). Meanwhile, *Ulva* sp. showed the highest N content and OM in addition to the low values for structural traits (i.e. the lowest carbon and fiber content), probably making it the most palatable species. Thus, it was the most consumed macrophyte in all the feeding assays, but this was only statistically significant under CDHA (Figures 5A and C).

Most macroalgae are usually more consumed than seagrasses, since the latter may have a lower palatability because of their high fiber content and lower nutritional quality (Valiela, 1995). However, some authors (Valentine and Heck 1999) have argued that seagrasses might actually have an intermediate palatability among several species of algae. Although in this work only a single macroalga species (i.e. *Ulva* sp.) was used, our study is in agreement with the intermediate palatability suggested for seagrasses. Within their intermediate palatability, the combination of those different traits related with palatability displayed by *C. nodosa* and *Z. noltei* from inner and outer bay (Figure 2), demonstrated to differently influence the consumption rate by *P. lividus*. For instance, while no intra-specific differences were found in the consumption of ZnI and ZnO, a significantly lower consumption was recorded in CnI *vs.* CnO. Intra-specific differences between *C. nodosa* plants were also found in structural traits, with plants inhabiting the inner bay displaying higher carbon content ($p < 0.001$). We also recorded a negative correlation between C content and consumption rate in the macrophytes assayed (-0.89, $p < 0.05$; Table 2), so it seems that structural traits may be an important driving force determining intra-specific differences in seagrass palatability.

However, similar levels of intra-specific variability were recorded in *Z. noltei*, but this variability did not translate into differences in consumption rates by *P. lividus*. This can be explained by two different arguments. First, values for morphological (i.e. width and thickness) and structural (i.e. carbon and fiber content) traits were higher in *C. nodosa* than in *Z. noltei* plants. Values recorded for *Z. noltei* from both locations were always below the lower

range recorded for *C. nodosa* (Figure 2). Hence, although intra-specific differences were found in the case of *Z. noltei*, they may not have surpassed the threshold values needed to reduce the feeding rate of *P. lividus*. Second, the levels of chemical defences in plants have also been commonly pointed out as an important factor determining food choice by most herbivores in terrestrial, freshwater and marine systems (Bolser and Hay, 1996; Cebrian et al., 2009; Mattson, 1980; Prusak et al., 2005). Intra- and inter-specific differences in the composition and concentration of these compounds have been measured in both seagrass species in the studied area (Suárez, 2010; Grignon-Dubois et al., 2012; Jiménez-Ramos et al., 2017; Manck et al., 2017). *P. lividus* has the ability to avoid plant species that synthesize toxic components or secondary dissuasive metabolites (Frantzis and Gremare, 1993). For instance, Lemee et al. (1996) observed no consumption of *Caulerpa taxifolia* by *P. lividus* during the summer, which is when higher deterrent concentrations occur in this macroalga. However, in winter and spring the production of secondary metabolites declines and *C. taxifolia* was found to be moderately consumed by *P. lividus*. Other studies have found selective behaviour of *P. lividus* with respect to *Posidonia oceanica* leaves with low deterrents and high nutritional quality (Vergés et al., 2011, 2007b). Thus, although our work was not able to assess the effect of chemical defences on consumption rates, past studies have found higher concentrations of phenolic compounds for *C. nodosa* and *Z. noltei* from the inner bay compared to those found in the outer bay (Grignon-Dubois et al., 2012; Jiménez-Ramos et al., 2017; Manck et al., 2017).

On the other hand, it should be noted that in our study, we did not discriminate between genotypic and phenotypic (environmental acclimation) differences in plant traits. Thus, the differences recorded in the studied plant variables can be the sum of both environmental acclimation and genetic differences (Koricheva, 2002; Martínez-Crego et al., 2016). Several studies on terrestrial and marine plants have reported different herbivory patterns between different genotypes, which were driven by differences in plant traits (Bailey et al., 2004; Tomas et al., 2011). In this way, past studies have shown that *C. nodosa* populations in Cadiz bay (Alberto et al., 2005) bear a high genetic variability, while others have also demonstrated that environmental differences may modify traits related to palatability (de los Santos et al., 2013), also including the synthesis of natural products (Manck et al., 2017). Therefore, both genetic variability and the acclimation to such different environmental conditions may explain the differences in traits that we observed between plants from the inner and outer bay, and thus both may contribute to the differences in the recorded consumption rates.

Our results also show that the higher consumption of *Ulva* sp. over seagrasses was only significant under CDHA (i.e. choice assays with abundant food). Boudouresque and Verlaque (2001) observed under field conditions that *P. lividus* showed selective preference for

different food sources, and was able to move from a preferred but rare resource to a less preferred but abundant one. However, these authors were unable to indicate the reasons responsible for such feeding choices and whether food availability played any role in such decision. Although our experimental design does not allow us to argue conclusively about the foraging behaviour of *P. lividus*, we have observed that they moved around the aquarium and were able to make choices among the different food sources, selecting the most palatable ones under high food availability. Thus, our results highlight the importance of developing studies using mixed diets and different food abundances.

Conclusions

This work shows that intra- and inter-specific differences between seagrass species are important factors that could alter seagrass consumption within the community, as demonstrated in *C. nodosa*. Structural traits seemed to be the main driver of *P. lividus* selection when seagrasses are the food choice groups with intermediate palatability, although the presence of natural products can be also important. We have also shown evidence that species diversity (species richness and abundance) can generate patterns where less palatable food choices are more consumed than more palatable ones (i.e. higher nutritional content and lower structural defences) as it was in the case of *Ulva* sp., especially when *P. lividus* is supplied with abundant food availability. Finally, these findings highlight the need to develop more in-depth studies on the influence of the macrophyte traits affecting palatability, which also consider the foraging behaviour of the herbivores. They also stress the importance of managing diversity in seagrass communities to improve the resistance and the resilience of seagrass ecosystems (Duffy et al., 2007).

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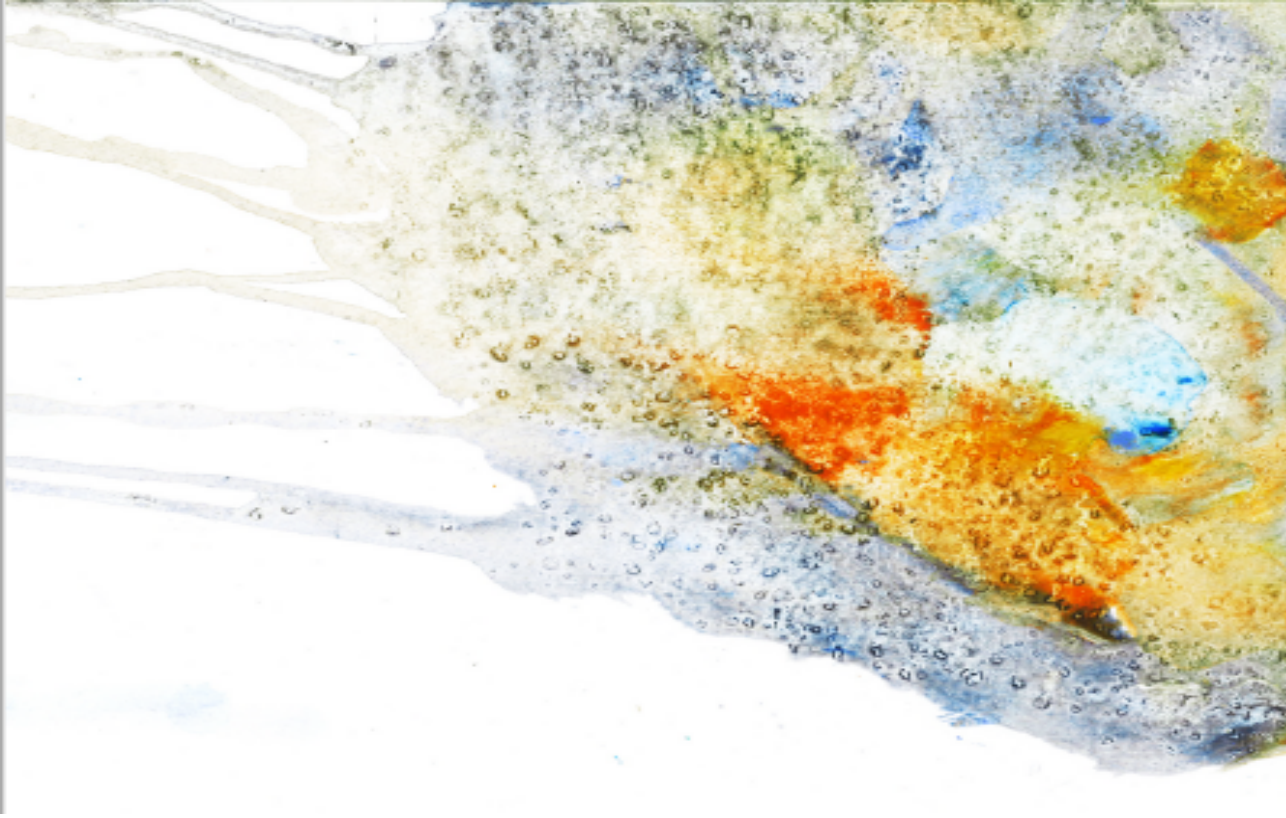

CHAPTER 4

Eutrophication and epiphytes are drivers of increased herbivory on seagrasses

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Eutrophication and epiphytes are drivers of increased herbivory on seagrasses

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Abstract

Eutrophication is one of the major threats for seagrass promoting effects in different compartments of the community (e.g. plants, epiphytes, fauna). Understanding the direction and strength of the different interrelationships is necessary not only to undertake an effective management but also in order to gain predictive capacity of the likely outcomes. In this study we: (1) investigate the leaf traits changes of *Cymodocea nodosa* after field acclimation to nutrient enrichment (2) explore the eutrophication consequences in feeding preference of *Paracentrotus lividus* by food and agar choice experiments. Results showed that eutrophication increase *C. nodosa* consumption by rising nutritional quality (higher N content), reducing structural and biomechanical traits and boosting epiphytes growth in leaves. On the other hand, others factors such as ephemeral macroalgae growth (*Ulva* sp.) may also affect the intensity of seagrass herbivory. Hence, this study improves our understanding of the implications of eutrophication on herbivory in seagrass ecosystems.

Introduction

Coastal eutrophication is one of the main factors leading to the decline of seagrass populations because of the direct (e.g. toxicity effects by nutrients) and indirect effects (e.g. shading effects by epiphytes growth) it promotes (Orth et al. 1984; Van Katwijk et al. 1997; Brun et al. 2002, 2008; Coll et al. 2011). In addition to negative effects, higher nutrients availability under eutrophication events raise the nitrogen content of plant tissues (Invers et al. 2004), yielding a greater nutritional quality. As a consequence, some studies have recorded a higher palatability of seagrass N-enriched leaves, and therefore higher consumption rates by herbivores (Cebrián and Lartigue 2004; Heck and Valentine 2006; Cebrián et al., 2009). However, experimental evidences do not completely support such nutritional hypothesis. While some studies found a positive correlation between leaf nitrogen content and its consumption rate by herbivores (Bjorndal 1980; Zieman et al. 1984; McGlathery 1995; Goecker et al. 2005), others reported the opposite pattern (Mariani and Alcoverro 1999; Kirsch et al. 2002; White et al. 2011). Moreover, recent studies have showed how seagrass fertilization significantly reduces the strength of the leaves (i.e., weaker leaves) (La Nafie et al., 2012, 2013), while other authors have hypothesized that this reduction may result in a higher consumption as leaves are easier to bite by herbivores (De Los Santos et al. 2012; Tomas et al. 2015). Hence, these findings have impaired our ability to elucidate the existence of clear patterns in seagrass herbivory and its relationships with plant traits.

On the other hand, eutrophication often leads to the proliferation of ephemeral macroalgae, such as *Ulva* sp. (Burkholder 2007; Chávez-Sánchez et al. 2017) or epiphytes which can outcompete seagrasses for light and nutrients (Dennison et al. 1993; Hauxwell et al. 2001), which further increase the susceptibility of seagrasses to such harsh conditions. Epiphytes are also a key element in the relationship between herbivores and seagrasses since their external location in the leaves may increase the feeding selectivity by herbivores (Heck and Valentine 2006; Marco-Méndez et al. 2015), because of the higher nutritional quality and the lower structural and morphological defenses they bear (Alcoverro et al. 2000). Hence, epiphytes wrapping leaves can strongly influence herbivore consumption on seagrass ecosystems.

In this framework, the main aims of this work were (i) to analyse the impact of eutrophication in the consumption rates of seagrass leaves; (ii) to determine the relative influence of nutritional, morphological, structural and biomechanical traits in this response and (iii) to assess the role played by epiphytes in the feeding choice done by the generalist consumer *Paracentrotus lividus* (sea urchin). In addition, we tested how the presence of a frequent and highly palatable species during coastal eutrophication events (i.e. *Ulva* sp.) influences the feeding selection done by *P. lividus*.

Materials and methods

Experimental setup

An *in-situ* nutrient enrichment experiment was carried out in an inner section of Cadiz bay (36º1'N; 06º15'W) over 3 months, from May to July, corresponding with the period of maximum growth and biomass of *Cymodocea nodosa* in this area (Peralta et al. 2008). In the water column, nutrient peaks usually occur in winter, with values up to 1.4 μM NO_2^-, 12 μM NO_3^-, 25 μM NH_4^+ and 1.5 μM PO_4^{3-} (Tovar et al. 2000). Two treatments (control and enriched plots) from now called experimental plots, consisting of three replicates each were distributed haphazardly in a large underwater *C. nodosa* meadow. Experimental plots (50x50cm) were separated by at least ten meters each and delimited by 8 sticks placed within the canopy of the seagrass bed. At the top of each stick (15 cm above the seafloor), a small mesh bag filled with slow-release fertilizer (Osmocote™; N:P:K; 18:9:3) was employed to alter nutrient levels in the water column. An empty mesh bag was attached to the sticks of the 3 control replicates representing ambient conditions. In each enriched plot 80 g of Osmocote® was applied (0.5 kg m^{-2}) distributed in each mesh bag equally. Water samples were collected weekly in the central part of the square at 15 cm of the seafloor using a silicone tube joined to sterilized plastic syringes and filtered through Whatman GF/F filters (0.45μm) to measure nutrient availability. At this moment, mesh bags containing Osmocote® were checked and replaced, in the case that some of them were missing. After 3 months, above and belowground biomass was gathered manually using a 400 cm^2 quadrat placed in the center of the experimental plots. Care was taken to collect shoots with intact vertical rhizomes to minimize chemical changes occurring over time in detached plants. *Ulva* sp. was also collected in the same area at the end of July. In the field and during transportation, all material was kept with water and aeration. Once in the laboratory, all seagrasses were kept in separated 24 litres tanks filled with aerated natural seawater at room temperature (20ºC) until the beginning of the experiment, *C. nodosa* plants were carefully selected according to its healthy appearance (no necrosis or bites). Moreover, epiphyte biomass from control and enriched plants were measured in 10 independent shoots.

Morphological analyses

In 5 independent samples from the initial pool of control and enriched plants, total length, width and thickness of *C. nodosa* leaves were determined with a digital caliper (Mitutoyo 500 AOS) and a thickness gauge (Mitutoyo 7301).

Nutritional and structural analyses

Nutritional content of control and enriched plants (with and without epiphytes) was examined in 5 independent samples from initial pool. For plants without epiphytes, *C. nodosa* leaves were cleaned of epiphytes carefully using a piece of soft paper. The water content in the samples was calculated as the weigh difference between fresh and dried biomass after 48 hours in the oven (60ºC).

Each sample was freeze-dried and pulverized in a ball-grinder to determine the total C and N content in tissues and fibre content. Elemental analysis was performed in a Perkin-Elmer 2400 elemental analyzer, while fibre content was done using the method of Van Soest et al. (1991) modified by De Los Santos et al. (2012).

Biomechanical analyses

Biomechanical properties of control and enriched plants were measured with an universal testing machine (Instron model 5542) and the BlueHill® software (v.2.18). The leaves were measured within 2d of sampling from initial pool, and the specimens were tested in the same sequence that they were collected so that the time of storage was homogeneous among samples and among treatments. Prior to the measurements, leaves were lightly and carefully cleaned of epiphytes and debris using a soft wet paper. The first outermost fully-developed leaf of the shoot was selected (normally the second youngest leaf), and a portion of the blade leaf (4 to 5 cm above the ligule, not including the leaf sheath) was cut for testing. Leaf-fracture properties were evaluated by cutting test, since this test measures the force required for foliar breakage (Wright and Vicent 1996; Aranwela et al. 1999), and were expressed at 2 levels: (1) total quantity of force needed to tear or cut a single leaf blade, which depends on the leaf size and its mechanical properties at the material level (F_{TA}, N); (2) material mechanical traits, normally called 'material properties', which are inherent properties of the material (F_{TS}; N·mm^{-2}). Regarding the ecological significance of these traits, whole-leaf mechanical traits indicate the force needed in absolute terms to cut or tear a single leaf blade by a herbivore, whereas material properties show the invested work or force required to ingest an amount of material, giving an idea of the cost-efficiency of the feeding process (De Los Santos et al. 2012). During the tests, a force to displacement curve was monitored. Since the whole leaf was cut transversally during the test, the force done to cut the lamina included the leaf veins (de Los Santos et al., 2012).

Collection of sea urchins

Sea urchins (*Paracentrotus lividus*) were collected in a nearby rocky shore, La Caleta, Cádiz (SW Spain, 36º31'39"N; 6º18'46"W). After permission from the local environmental

authorities, individuals were collected at a depth of 2 meters. Harvesting was carried out carefully by snorkelling, avoiding damage to the organisms. Sizes varied were between 3-5 cm in diameter (adult size). Collected individuals were kept in coolers with seawater and transported to the laboratory as soon as possible (i.e. within one hour). Once in laboratory, sea urchins were distributed in five tanks (30 litres; 18 individuals each) with aeration and were fed with *Ulva* sp. during 3 days before the starting of the assays to acclimate organisms to laboratory conditions.

Feeding preference assays

Feeding preference assays were designated to determine if eutrophication alters the feeding rate of *Paracentrotus lividus*, and whether this change is mediated by epiphyte load or by changes in those traits determining tissue palatability. In addition, to delve into the understanding of how different traits mediate sea urchin consumption, we added another source of food, the macroalgae *Ulva* sp, a very common macrophyte in coastal eutrophication events (Hernández et al. 1997; Burkholder et al. 2007). This macroalgae is highly consumed by the sea urchin (Boudouresque and Verlaque, 2001) since it has little structural defenses and a high nutritional quality and, thus it is highly palatable. This treatment serves as a control of the feeding capacity of the sea urchins along the different treatments. Thus, two different assays were performed:

i) *Individual food assays*: A total of five different treatments were designed for each type of food: *Ulva* sp. (U); control leaves with no epiphytes (CNE); control leaves with epiphytes (CE); enriched leaves with no epiphytes (ENE); enriched leaves with epiphytes (EE). In each replicate (n=6), a total of 6 g of each type of food (i.e. U, CNE, CE, ENE and EE) were placed independently into each aquarium (i.e. 30 individual food assays were performed in total). Simultaneously, autogenic controls (i.e. fresh biomass of *Ulva* sp. and seagrass not exposed to sea-urchins) were performed to account for potential changes not due to grazing. Results showed no significant autogenic changes in *Ulva* sp. and seagrass during control tests conducted and were thus not considered further in the analysis (t_U = 1.18, df = 23, $p > 0.05$; t_{CNE} = -0.35, df = 23, $p > 0.05$; t_{CE} = -1.55, df = 23, $p > 0.05$; t_{EE} = –0.45, df = 23, $p > 0.05$; t_{ENE} = -1.12, df = 23, $p > 0.05$).

ii) *Paired food assays:* In addition to individual food assays, a paired food assay was performed between *Ulva* sp. and the different types of *C. nodosa* leaves (CNE, CE, EE, ENE), to test how the presence of a highly palatable and common food source in coastal eutrophication events, such as *Ulva* sp., influences the consumption rates over seagrass leaves. A total of 3 g of fresh *Ulva* sp. and 3 g of each one of the fresh seagrass leaves coming from the different treatments (i.e. CNE, CE, EE, ENE) were placed in the aquaria together

(U+CNE; U+CE; U+EE; U+ENE), resulting in 24 paired food assays. Concurrently, autogenic controls were performed to account for potential changes not due to grazing. Results showed no significant autogenic changes in *Ulva* sp. and seagrass during control tests conducted and were thus not considered further in the analysis.

Experiments were conducted on consecutive days for each feeding assay (individual and paired food assays) due to time and space restrictions. Six replicates were done for each feeding assay and were conducted over a 24h period. Three sea urchins were used per aquarium and were renewed by new ones coming from the reservoir for each new trial. All sea urchins were starved during 24 h prior to being used in the experiment. Each aquarium contained 24l of seawater with constant aeration and the bottom was covered with sand. Both *Ulva* sp. and seagrass biomass were distributed randomly and secured to the bottom. Seawater temperature varied between 21 and 23 ºC under an 8:16 h photoperiod, because *P. lividus* usually exhibit a nocturnal activity (Bouderesque *et al.*, 2001). Once experimental time ended (24 h), the remaining material was removed from each aquaria, blot-dried, and weighed and consumption rates were calculated as the difference between initial and final biomass per sea urchin and day (i.e. g wet weight individual^{-1}d^{-1}).

Agar preference assays

Agar diets were used to test the capacity of consumers to detect differences in plant nutritional features since the structural characteristics are eliminated in this type of diet (Ojeda and Caceres 1993; Prado and Heck 2011). A total of 6 g of each type of food (i.e. U, CNE, CE, ENE and EE) were placed independently in a heated mixture of 100 ml of distilled water (as indicated by Goecker et al. 2005) and 2 g of agar (Carolina Biological Supply). The mixture was poured into small moulds (2 cm diameter) and allowed to cool for 1 h within a refrigerator. Samples were then removed from the moulds and weighed to the nearest 0.001 g. The experimental design was conducted as individual food assays with 6 replicates each one (i.e. five types of treatments: U; CNE; CE; EE; ENE). Autogenic controls were conducted simultaneously with agar-preference assays and indicated a significant decline in diet weight (t_U = 29.87, df = 23, p<0.05; t_{CNE} = 40.98, df = 23, p<0.05; t_{CE} = 40.67, df = 23, p<0.05; t_{EE} = 39.56, df = 23, p<0.05; t_{ENE} = 42.12 , df = 23, p<0.05) over a 24 h of the experimental period (ca. 4.5% of initial weight). In addition to autogenic controls, an agar diet control was also run (n=6), where under the same experimental conditions agar blocks without the addition of *Ulva* sp. or seagrass biomass were offered to the sea urchins. This control was run in order to discriminate whether the addition of ground macrophyte biomass or the agar by itself were the responsible of the change in the consumption rates of the sea urchins. Consumption rate was estimated as the difference in wet weight during 24h (g wet weight individual^{-1}d^{-1}) and taking into account the biomass loss in the agar blocks measured in the autogenic controls. As

described for the feeding preference assays, agar preference assays were conducted during consecutive days for each feeding assay (individual, paired and agar food assays).

Statistical analyses

Prior to any statistical analysis data were checked for normality (Shapiro-Wilk normality test) and homocedasticity (Bartlett test of homogeneity of variances test). Data were transformed (i.e. consumption rates in no food choice assays) when necessary to meet normal distribution assumptions. Differences in seawater nutrients concentration among treatments, epiphytic biomass in *C. nodosa* leaves in each preference assay and nutritional, structural and biomechanical properties of plants were analysed using a paired *t*-test. Differences in consumption rates were analysed using a 2-way ANOVA for no-choice assays (i.e. food-preference and agar-diet assays) and between *C. nodosa* consumption in paired food choice assays. Differences in consumption rate between *Ulva* sp. and *C. nodosa* leaves from the paired food choice assays were analyzed using a paired *t*-test. The amount of epiphytes in leaves from each treatment was analyses using a 1-way ANOVA. When significant differences were found a Tukey post-hoc test was applied. Data were presented as mean ± s.e. The significance level (α) set in all tests performed was 0.05. Statistical analyses were computed with R 3.0.2 (R Development Core Team 2013).

Results

Nutrients concentration in natural seawater

Nutrients concentration *in situ* differed significantly between enriched and control treatments along the experimental time, revealing large differences in nutrients availability for plant community depending on the experimental treatment. Enrichment increased significantly phosphate (0.37 ± 0.04 vs 0.83± 0.31 μM; t=-2.48, df=9, p=0.038) and ammonium concentrations (5.69 ± 3.43 vs 23.32 ± 6.68 μM; ; t=-2.37, df=9, p=0.041).

Leaf traits

Morphological traits varied significantly among control and enriched plants in terms of width and thickness. Enriched plants were significantly broader and thicker than control plants (Table 1). Nutritional quality of plants (i.e. N content) increased in enriched plants significantly, showing a 46.1% higher N content in leaves than the control plants ($p<0.01$).

Table 1. Leaf traits of *Cymodocea nodosa* leaves exposed to eutrophication conditions (ambient vs enrichment). Values are means ± s.e. of replicates. Bold letters indicate significant differences (p<0.05).

Variables	Control plants	Enriched plants	Statistical results
Morphological traits			
Lenght (cm)	42.6±2.01	40.46±3.06	t= 0.581; df= 28; p= 0.565
Width (mm)	3.52±0.13	4.09±0.08	**t= -3.584; df= 28; p= 0.001**
Thickness (mm)	0.17±0.003	0.22±0.003	**t= -8.901; df= 28; p< 0.001**
Nutritional traits			
Leaf Nitrogen (%)	2.07±0.07	4.30±0.20	**t= -10.171; df= 8; p< 0.001**
Rizhom Nitrogen (%)	0.96±0.14	1.28±0.19	t= -1.322; df= 8; p=0.222
Leaf C:N	17.66±0.86	8.74±0.58	**t= 9.152; df= 8; p< 0.001**
Rizhom C:N	45.74±6.53	33.63±3.66	t= 1.617; df= 8; p= 0.144
Structural traits			
Leaf Carbon (%)	31.22±0.47	31.96±0.89	t= -0.739; df= 8; p= 0.481
Rizhom Carbon (%)	34.76±0.82	34.59±0.47	t= 0.182; df= 8; p= 0.859
Fiber content (%)	45.62±0.58	42.73±0.70	**t= 3.065; df= 9; p= 0.013**
Biomechanical traits			
F_{TA} (N)	0.570 ± 0.075	0.229 ± 0.028	**t= 4.416; df= 18; p< 0.001**
F_{TS} (N/mm^2)	0.836 ± 0.098	0.299 ± 0.033	**t= 5.606; df= 18; p< 0.001**

The presence of epiphytes in *C. nodosa* leaves increased significantly their nutritional quality (p<0.01). Therefore, measuring the N content in leaves coated by epiphytes resulted in an increase of 2.20 ± 0.09 and 4.41 ± 0.18 % in control and enriched plants respectively. Regarding structural traits, fiber content was significantly higher in control plants, while no significant differences were recorded for C content in both leaves and rhizomes. Moreover, control plants bear the highest values of F_{TA} and F_{TS} (the whole-leaf and material mechanical traits), and therefore being the most resistant leaves (Table 1). Although an increase in the biomass of epiphytes in enriched leaves was found, there were no significant differences when compared to control leaves (8.48 ± 0.52 vs 10.36 ± 1.55 g DW ·shoot^{-1}, t = -2.70, df = 7, p = 0.072).

Feeding preference assays

Overall, *Ulva* sp. was the most consumed in both individual and paired food assays when compared to *C. nodosa* plants (Figures 1 and 2, Table 2).

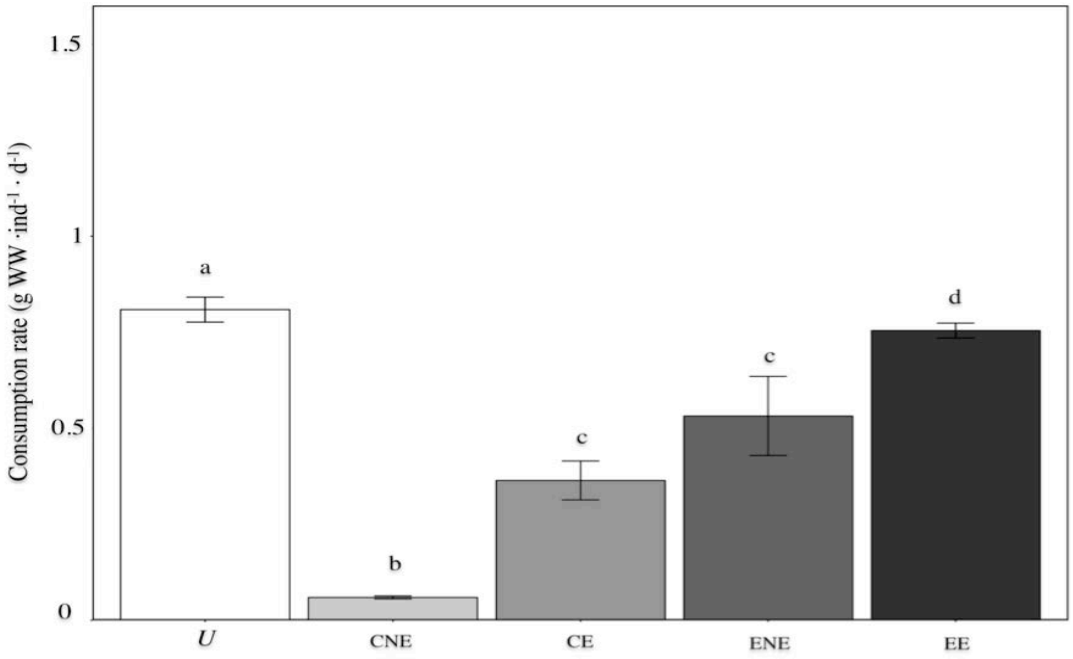

Figure 1. Consumption rates of *Ulva* sp. and *Cymodocea nodosa* plants (CNE: Control plants, no epiphytes; CE: Control plants, epiphytes; ENE: Enriched plants, no epiphytes; EE: Enriched plants, epiphytes) by sea urchin in no choice food preference assays (g wet weight [WW] ·ind^{-1} · d^{-1}; mean ± s.e.) (n=6). Letters indicate significant differences (p<0.05).

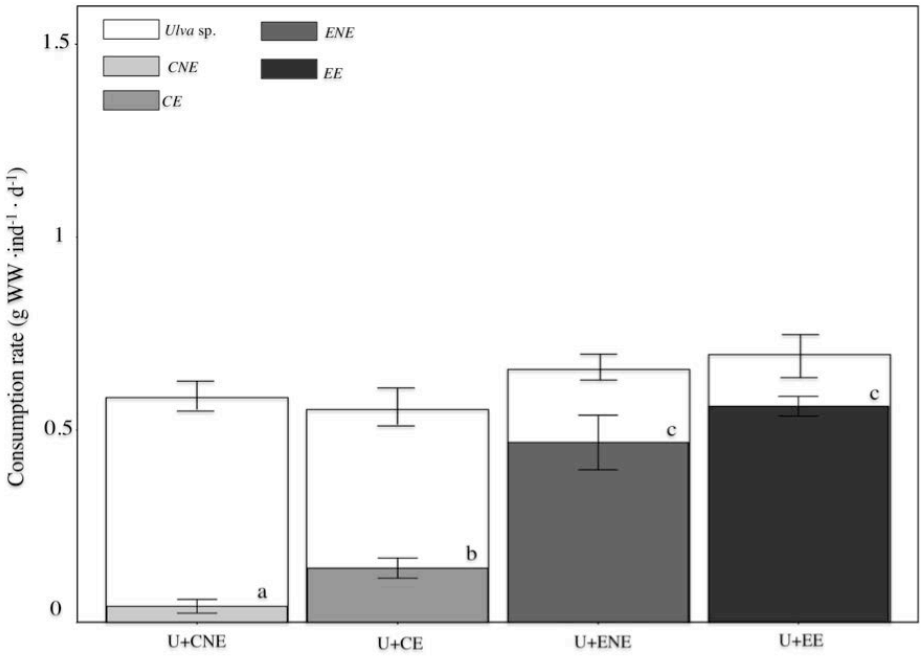

Figure 2. Consumption rates of *Ulva* sp. and different *Cymodocea nodosa* plants (CNE: Control plants, no epiphytes; CE: Control plants, epiphytes; ENE: Enriched plants, no epiphytes; EE: Enriched plants, epiphytes) by sea urchin in paired food choice pereference assays (g wet weight [WW] $\cdot ind^{-1} \cdot d^{-1}$; mean ± s.e.) ($n$=6). Letters indicate significant differences (p<0.05) among *C. nodosa* plants by ANOVA's test.

Table 2. Results from paired food assays with paired t-tests. Bold letters indicate significant differences (p<0.05). U: *Ulva* sp.; CNE: Control plants, no epiphytes; CE: Control plants, epiphytes; ENE: Enriched plants, no epiphytes; EE: Enriched plants, epiphytes.

Variables, factors	t	df	*p*
UxCNE	-15.81	4	**<0.01**
UxCE	-8.357	4	**0.001**
UxCE	-0.429	4	**0.048**
UxEE	-2.98	4	**0.040**

Regarding seagrass consumption, *Paracentrotus lividus* had the highest preference for both CE and EE plants in individual and paired food assays, showing a similar consumption pattern between both treatments (Table 3). The presence of epiphytes increased significantly the consumption over *C. nodosa* leaves, and especially in control plants (83.52% increase in individual food assays and 69.48% increase in paired food assay). Regarding enriched plants,

the presence of epiphytes had a minor effect in both individual (10.44% increase; $p<0.05$) and paired food (16.79% increase; non significant) assays. Otherwise, it is remarkable that the total consumption rate by *P. lividus* increased in paired food assays when compared to individual food assays (Figures 1 and 2).

Table 3. ANOVA 2-ways test results on consumption rates treatments for all preference assays. Bold values indicate significant differences ($p<0.05$).

Variables, factors	DF	MS	F	*p*
Individual food assays (log)				
Treatment	2	0.992	51.02	**<0.001**
Epiphytes	1	0.535	23.737	**<0.001**
Treatment x Epiphytes	1	0.378	0.579	**<0.001**
Error	10	0.01		
Paired food assays (sqrt)				
Treatment	1	0.538	106.61	**<0.001**
Epiphytes	1	0.028	5.587	**0.045**
Treatment x Epiphytes	1	0	0.003	0.957
Error	8	0.005		
Agar food assays				
Treatment	2	0.243	18.318	**<0.001**
Epiphytes	1	0.046	6.934	**0.025**
Treatment x Epiphytes	1	0.006	0.916	0.361
Error	10	0.006		

*Paired food assay analyses only among consumption rates of *C. nodosa* plants.

Agar preference assays

The consumption of agar blocks free of *Ulva* sp. or seagrass biomass was marginal (0.094 ± 0.027 gWW $\cdot ind^{-1} \cdot d^{-1}$), and significantly lower than the consumption of those agar blocks containing *C. nodosa* from control treatments (i.e. CNE; one-way ANOVA df=1; MS= 0.079; F= 35.46; $p=0.0039$), which indicates that the inclusion of *C. nodosa* leaves within the agar blocks encouraged their consumption.

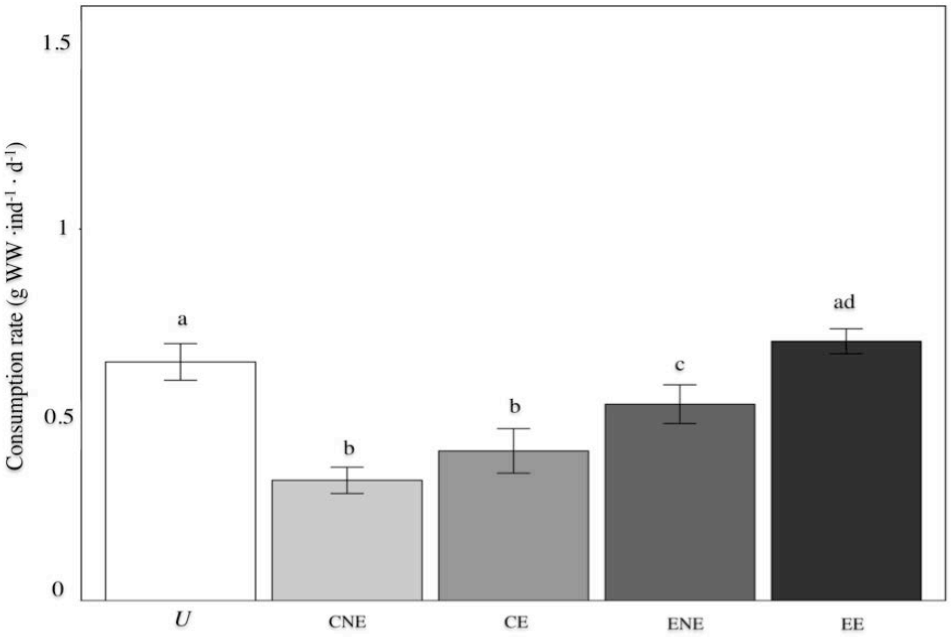

Figure 3. Consumption rates of *Ulva* sp. and *Cymodocea nodosa* plants (CNE: Control plants, no epiphytes; CE: Control plants, epiphytes; ENE: Enriched plants, no epiphytes; EE: Enriched plants, epiphytes) by sea urchin in agar diet (g wet weight [WW] $\cdot ind^{-1} \cdot d^{-1}$; mean ± s.e.) ($n$=6). Letters indicate significant differences (p<0.05).

When morphological, structural and biomechanical traits were removed by using the agar preference assays, *Paracentrotus lividus* maintained the same order of preferences as in previous assays using intact vegetal material (i.e. leaves and *Ulva* sp.). *Ulva* sp. was the most consumed, followed by EE plants while CNE treatment was the less consumed (Figure 3 and Table 3). However, it is important to note the significant increase in consumption rate recorded in control plants (CNE) with regard to food preference assays, resulting in a significant 81.53 ± 6.06% higher consumption rate than CNE in individual food assays (p = 0.0071).

Discussion

Our results clearly indicate that eutrophication alter the rate of consumption of *Cymodocea nodosa* leaves by two different but complementary ways: by increasing the nutritional quality of the leaves, and by increasing the presence of epiphytes. However, epiphytes presence by itself had a key role increasing greatly the consumption rate even of control leaves bearing low palatability.

Plants subjected to eutrophication during three months increased the N content of their leaves, which improves their nutritional quality (Table 1). It makes plants more palatable to herbivores (Cebrián et al. 2009), and as a consequence herbivores increase the consumption over these tissues, as demonstrated in this work (Figures 1, 2 and 3). The nutritional quality is considered the main factor regulating feeding decisions by consumers in terrestrial and aquatic ecosystems (Elser et al. 2000; Cebrián and Lartigue 2004). For instance, Prado and Heck (2011), observed in a combined diet experiment using different species of seagrasses, that sea urchins selected seagrass tissues primarily based on nutritional characteristics, even when differences among dietary components were relatively low. In addition to improving the nutritional quality of the leaves, eutrophication reduced the structural and biomechanical defenses of the leaves. Enriched plants had significantly weaker leaves and a lower fiber content (Table 1), as demonstrated in previous studies (La Nafie et al. 2013; de los Santos et al. 2013; Soisson et al. 2017). Hence, our results showed that the increase of the nutritional quality combined with the lower fiber content and weaker leaves in those plants subjected to nutrient fertilization, increased significantly the consumption of *C. nodosa* leaves in both food preference assays (Figures 1 and 2). On the other hand, agar preference assays revealed the importance of morphological, structural and biomechanical traits in the feeding preference of this generalist herbivore, since a significant increase in consumption was found in grounded control plants (CNE) when compared to the consumption recorded in intact leaves and when compared to agar blocks without seagrass biomass (Figures 1 and 3). Although the generalization of this finding is constrained by the type of consumer, our results suggest that nutritional differences among seagrass leaves are not the unique driving force in the selection done by *P. lividus*. Thus, this result emphasizes the importance of the structural or mechanical components as key factors to understand seagrass consumption as already suggested in some previous works (Mariani and Alcoverro 1999; Vergés et al. 2007; De Los Santos et al. 2012)

The presence of epiphytes increased significantly the consumption of seagrass leaves independently of their nutritional status (i.e. CNE and ENE). This enhanced consumption rate can be also explained by the higher nutritional quality of the tandem formed by seagrass leaves plus epiphytes, since the N content in leaves with attached epiphytes (i.e. CE and EE) was significantly higher than in those leaves where epiphytes were removed ($p<0.01$). Hence, this preference for leaves coated by epiphytes vs. leaves without epiphytes confirms those previous findings indicating that epiphytes strongly influence consumption rates and feeding choice preference done by herbivores (Greenway 1995; Marco-Méndez et al. 2012).

In addition, it is noteworthy that sea urchins consumed significantly more *Ulva* sp. than seagrasses in all the assays, which might by due to the higher nutritional value and the

lower structural protection (e.g. fiber and carbon content…) that bears this macroalga. Moreover, total sea urchin consumption is higher in paired food assays, since the presence of a combination of palatable food sources (i.e. *Ulva* sp. and *C. nodosa*) uses to increase its daily rate of consumption, as it has been shown in previous field studies (Boudouresque and Verlaque, 2001). This result has great ecological implications, since during eutrophication events it has been previously shown that plants are subjected to indirect (e.g. light reduction promoted by ephemeral algae growth, anoxic conditions, etc) and direct effects (e.g. ammonium toxicity) affecting negatively to their growth and survival (Brun et al., 2003, 2002; Burkholder et al. 2007), and our study demonstrates that eutrophication may also increase the consumption of seagrass leaves, which may further strengthen the negative effects promoted by eutrophication. On the other hand, this study also indicated the importance of the proliferation of other ephemeral macroalgae, such as the genus *Ulva* during eutrophication events, which tend to be more palatable to consumers and may reduce the direct consumption over seagrasses. Therefore their presence may improve the resilience of seagrass plants under eutrophication, as previously suggested by Moreno-Marín et al. (2016), when analysing the direct toxic effect of ammonium.

Conclusions

The present study showed that eutrophication events increase *C. nodosa* palatability by different and complementary ways: i) by rising nutritional quality (i.e. higher N content); ii) by decreasing structural and biomechanical properties of leaves and iii) by boosting epiphytes growth in leaves. Moreover, the sea urchin preference patterns seem to respond to the proliferation of other macroalgae with more palatability (i.e. *Ulva* sp.), which lead the *Paracentrotus lividus* consumption over them and may improve seagrass resilience under eutrophication disturbance.

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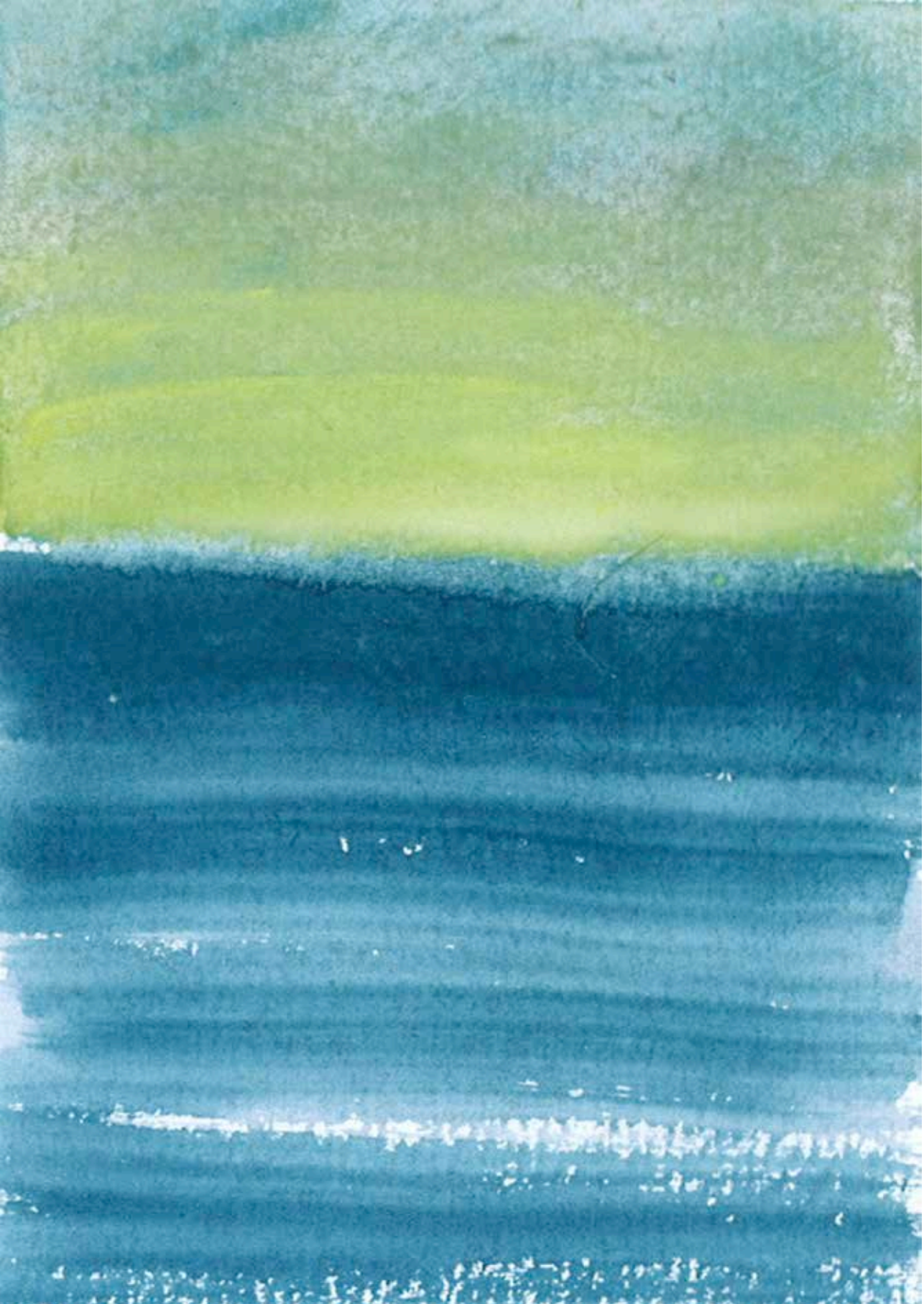

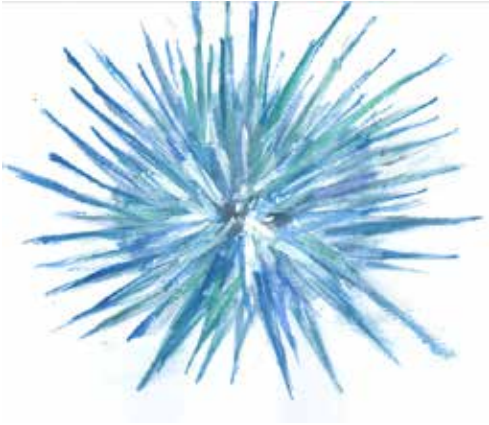

CHAPTER 5

Global and local disturbances interact to modify seagrass palatability

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Lo que tu haces marca una diferencia. Así que debes decidir qué tipo de diferencia quieres marcar en el mundo.
What you do makes a difference, and you have to decide what kind of difference you want to make.

Jane Goodall

Global and local disturbances interact to modify seagrass palatability

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Abstract

Global change, such as warming and ocean acidification, and local anthropogenic disturbances, such as eutrophication, can have profound impacts on marine organisms. However, we are far from being able to predict the outcome of multiple interacting disturbances on seagrass communities. Herbivores are key in determining plant community structure and the transfer of energy up the food web. Global and local disturbances may alter the ecological role of herbivory by modifying leaf palatability (i.e. leaf traits) and consequently, the feeding patterns of herbivores. This study evaluates the main and interactive effects of factors related to global change (i.e. elevated temperature, lower pH levels and associated ocean acidification) and local disturbance (i.e. eutrophication through ammonium enrichment) on a broad spectrum of leaf traits using the temperate seagrass *Cymodocea nodosa*, including structural, nutritional, biomechanical and chemical traits. The effect of these traits on the consumption rates of the generalist herbivore *Paracentrotus lividus* (purple sea urchin) is evaluated. The three disturbances of warming, low pH level and eutrophication, alone and in combination, increased the consumption rate of seagrass by modifying all leaf traits. Leaf nutritional quality, measured as nitrogen content, was positively correlated to consumption rate. In contrast, a negative correlation was found between feeding decisions by sea urchins and structural, biomechanical and chemical leaf traits. In addition, a notable accomplishment of this work is the identification of phenolic compounds not previously reported for *C. nodosa.* Our results suggest that global and local disturbances may trigger a major shift in the herbivory of seagrass communities, with important implications for the resilience of seagrass ecosystems.

Introduction

Human activity is increasing, at the rate of 0.4% yr^{-1}, the concentration of CO_2 in the atmosphere, which is expected to double from preindustrial levels by the middle of this century (Houghton 2001). Approximately 30 % of CO_2 emissions are absorbed by marine waters; therefore, marine species have to cope with increasing ocean acidification in combination with rising temperatures and other anthropogenic disturbance (i.e. a physical force, agent, or process, either abiotic or biotic, causing a perturbation in a ecological component or system; (Rykiel Jr 1985) and references therein), such as eutrophication. In this context, coastal vegetated ecosystems are one of the most threatened (Polunin 2008). Within coastal vegetated habitats, seagrasses form the basis of one of the most species-rich and relevant ecosystems (Short et al. 2011), but also one of the most threatened (Waycott et al. 2009). Seagrass communities are under threat from co-occurring global (e.g. rising temperatures, ocean acidification) and local disturbances (e.g. eutrophication), which are already acting together in many coastal areas with effects expected to increase in the near future (Nicholls et al. 2007; Halpern et al. 2015). How these changes will affect seagrass-herbivore interactions is one of the main questions for future seagrass research. We are far from being able to predict the outcome of interacting multiple perturbations (i.e. the response of an ecological component or system to disturbance by deviations in the values describing the properties of the component or system' relative to a specified reference condition; (Rykiel Jr 1985) and references therein) on the functioning of seagrass ecosystems. Until now, mesocosm and field experiments, mostly addressing only one factor at a time (Short and Neckles 1999; Burkholder et al. 2007; Koch et al. 2013) but some with multifactorial designs (De Los Santos et al. 2009; La Nafie et al. 2013; Villazán et al. 2016), have shown the direct effects on plants in terms of growth and production, and changes in leaf content (N, P, non-structural carbohydrates) (Short and Neckles 1999; Burkholder et al. 2007). Even though several recent studies have analyzed the indirect effects of seagrass-herbivore interactions (Connell and Russell 2010; Burnell et al. 2013; Campbell and Fourqurean 2013; Tomas et al. 2015), few have addressed the outcome of interactions between more than two factors.

Changes in the growth or content of seagrass leaves due to global and local disturbances can exert a significant impact on seagrass communities because these changes may influence leaf palatability, and thus make plants more or less vulnerable to herbivores (Cebrián and Duarte 1998; Goecker et al. 2005; Prado and Heck 2011). Eutrophication, one of the main local causes of seagrass decline, has been shown to affect the intensity of herbivory in seagrass ecosystems (Cebrián and Lartigue 2004; Heck and Valentine 2006). Under elevated nutrient concentrations, plants may increase the nutritional quality of their leaves (i.e. higher N content; (Van Katwijk et al. 1999; Villazán et al. 2013a; b)). However, the responses to this change may not always be straightforward. For example, Goecker et al.

(2005) and Prado and Heck (2011) have shown that generalist herbivores (parrotfish and sea urchins) preferentially feed on leaves with higher nitrogen content, and lower leaf toughness and fiber content. However, others studies have reported the opposite trend (Mariani and Alcoverro 1999; Kirsch et al. 2002; White et al. 2011).

At the global scale, increasing CO_2 concentration may increase both seagrass primary productivity and leaf C:N ratio (Hughes and Bazzaz 1997; Koch et al. 2013), which may reduce the palatability and nutritional quality of leaves. However, elevated CO_2 concentrations in combination with ocean acidification has also been shown to down-regulate the production of C-based phenolic compounds in marine plants and thus enhance herbivore consumption of seagrass tissues (Arnold et al. 2012), which may counterbalance the aforementioned positive effects on seagrasses. Warming temperatures are also predicted to increase rates of primary production (Raven and Geider 1988), alter C:N ratios (Staehr and Wernberg 2009) and/ or the production of defensive secondary metabolites (Sudatti et al. 2011), which may further modify leaf palatability. Understanding how changes in the abiotic environment can modify the outcome of species interactions and being able to scale up these changes to the community level is of paramount importance for ecologists, particularly for interactions involving keystone species that have disproportionately large effects on community and habitat structure (Harley et al. 2006; Kordas et al. 2011). This challenge requires a multi-scale approach from the level of individual responses to that of populations, communities and the entire ecosystem (Russell et al. 2012), taking into account that the overall effect of multiple stressors can be non-additive (Woodward et al. 2010; Villazán et al. 2016).

Cymodocea nodosa (Ucria) Ascherson is a seagrass distributed across the Mediterranean Sea and adjacent eastern Atlantic coasts (Ruiz et al. 2015), including the Macaronesian archipelagos of Madeira and the Canaries, all the way down to Senegal in the western African coast (Alberto et al. 2006; Mascaró et al. 2009). These meadows constituted by *C. nodosa* are often the dominant vegetated communities on shallow soft substrates of the Andalusian coasts (south Spain), co-habiting with *Posidonia oceanica* or *Zostera noltei.* Interestingly, the experimental site of this work, Cádiz bay, is the unique place in Europe where the dominant vegetated community of *C. nodosa* (total extension of 1200 ha) is co-habiting with *Z. noltei* and *Zostera marina* populations (Brun et al. 2015). These communities are highly productive and providing food and shelter for diverse invertebrates and fish assemblages (Rueda et al. 2001; López de La Rosa et al. 2006). Moreover, the trophic importance of *C. nodosa* as a food resource for herbivores were observed in several (Cebrián et al. 1996; Fernandez et al. 2012; Del Río et al. 2016).

The main aims of this work are two-fold: to study the direct response of seagrasses to the main drivers of change in their communities, warming, acidification and eutrophication (Waycott et al. 2009); and to determine how these drivers indirectly affect the feeding preference of herbivores. We used a full-factorial experimental design to test for the effects of seawater temperature, pH and NH_4^+ concentration on traits of *C. nodosa* plants. After this, the plants were offered to *Paracentrotus lividus*, which is a type of purple sea urchin and a generalist herbivore of seagrass meadows, in individual feeding assays (i.e. just one food source) or in combined diet assays with a highly palatable species (*Ulva* sp.). Leaf traits and the consumption rate by sea urchins were measured to determine the main factors driving the feeding choice of this generalist herbivore.

Materials and methods

Sampling site and plant acclimation period

The seagrass *Cymodocea nodosa* Ucria (Ascherson) was randomly collected in the fall from large submerged meadows found in inner Cádiz Bay (36°28'09.08"N—06°15'04.64"W, South Spain; (Brun et al. 2015). *C. nodosa* inhabits the shallow south-western area along the intertidal border and in continuous monospecific meadows in the subtidal zone, which are found at 0.4 m above and 0.5 m below the chart datum of lowest astronomical tide, respectively (Olivé et al. 2013). Plant biomass were selected in the field and collected from different patches in a large area (~150 × 150 m), to ensure the genetic independence of plants, since in this area the sexual reproduction is as high as clonal ones (Alberto et al. 2005). Plants were transported to the laboratory at the University of Cádiz, southern Spain, within 2 h of collection in an ice chest. Upon arrival, experimental plant units (EPUs) of *C. nodosa,* consisting of one vertical shoot with its first rhizome segment (i.e. first internode), were carefully selected for a healthy appearance. Then 20 EPUs of healthy *C. nodosa* were allocated to each of 24 transparent incubation chambers (1.5 L) containing natural sandy sediment. Chambers were connected to seawater reservoirs (65 L) (three chambers per reservoir) by silicon tubs. The seawater reservoir received sand-filtered seawater at a rate of 6 L d^{-1}. Water in the incubation chambers was renewed by pumping water from their respective reservoir at a rate of 1.5 L d^{-1} in an open system. The water within chambers was homogenized by using air inlet drops.

After five days of plant acclimation to laboratory conditions (natural seawater, ambient light and temperature), seawater parameters (temperature, NH_4^+ and CO_2 concentration) were modified in the different seawater reservoirs (65 L) in a full factorial experimental design combining three of the main factors that are currently acting together in coastal areas (i.e. warming, acidification and NH_4^+ enrichment) in order

to determine how these drivers affect the leaf traits of the temperate seagrass *C. nodosa*. A three-full factorial experiment was conducted during a month. Two temperature levels were used: (a) the local temperature treatment with ambient temperature ca. 22 ºC and (b) the high temperature treatment with seawater heated to 4 °C above ambient levels until ca. 26 ºC. We also used two pH levels: (a) the current pH treatment of ca. 8.12, equivalent to ca. 415 ppm CO_2 and (b) the forecasted pH treatment ca. 7.69, equivalent to future conditions of ca. 720 ppm CO_2. Finally, we used two NH_4^+ levels: (a) the ambient NH_4^+ treatment with no added NH_4^+ and (b) the enriched NH_4^+ treatment where NH_4^+ was added to keep the concentration ca. 31 μM NH_4^+. All treatment levels were found in all combinations thus rendering a fully orthogonal design (8 treatment combinations in total). Each treatment combination was replicated 3 times resulting in a total of 24 experimental chambers. The levels of temperature and pH were selected according to the scenario forecasted by the Intergovernmental Panel on Climate Change (IPCC).

Temperatures were maintained by recirculating water through a heater (Tetra HT 100W). The pH in reservoirs with forecasted pH values were obtained by adding small amounts of HCl (0.01N) to the seawater until reaching the pH values linked to forecast ppm of CO_2 (ca. 720 ppm total scale) (e.g. (Netten et al. 2013)). The NH_4^+ enrichment was obtained by daily addition of NH_4^+ to the reservoir from a NH_4^+ stock solution to keep the concentrations as close to the target concentration as possible (ca. 31 μM NH_4^+). The ammonium addition corresponded to ca. 700 μmol g FW^{-1} d^{-1} in enriched NH_4^+ chambers. The concentration of ammonium was monitored according to Invers *et al.* (2004) (Invers et al. 2004) every two–three days in all chambers and daily in all reservoirs. Water samples were collected ten minutes after addition of ammonium. Seawater temperature and pH in the incubation chambers were measured daily at sunrise and sunset. The average salinity in the chambers during the experimental period was 30.08 ± 0.11. Light period was 16:8 h (light:darkness) with an instant photon flux of 325 μmol photon m^{-2} s^{-1}. S1 Table lists the total alkalinity, pH, temperature, as well as the dissolved inorganic carbon in the seawater in the incubation chambers.

At the end of the cultivation period (1 month), plant samples from each treatment were split: 5 EPUs from each chamber were selected for structural analyses (i.e. length, width and thickness) while another 5 EPUs from each chamber were collected for biomechanical measurements (i.e. leaf fracture properties) and once measured, immediately frozen at –18°C for complementary nutrient analyses (i.e. N, C and fiber content). Additionally 6 g of fresh leaf tissue from each chamber were immediately frozen at -80ºC for chemical analyses (i.e.

natural product determination). The remaining plants were kept alive in a 24 L tank with aeration and natural sandy sediment for feeding trials.

Structural and nutritional analyses

Leaf structural measurements were taken from healthy and fully-developed leaves from 5 EPUs per each chamber at the end of cultivation period. Each sample was freeze-dried and pulverized in a ball-grinder. Leaf thickness was measured 2 cm above the ligule using a thickness gauge (mm). The total C and N content in tissue samples was measured using a Perkin-Elmer 2400 elemental analyser. Nutrient content of tissues were examined in molar ratios, and the final results were based on dry biomass. Intracellular concentrations of NH_4^+ were measured on duplicated leaf samples from each aquarium. Samples were rinsed in deionized water and ca. 0.5 g (FW) of the tissue was ground in 20 ml of boiling deionized water (Dortch et al. 1984). Samples were sonicated for 10 min and then centrifuged for 20 min at 5,000 g. Finally, the concentration of NH_4^+ was measured in the supernatant following Grasshoff et al. (2007).

The dry biomass of each sample was used to determinate fibre content using a method modified from Van Soest et al. (1991) (Van Soest et al. 1991). Namely, approximately 30 mg samples of dry biomass were boiled in 2 ml of neutral detergent for 1h. Subsequently, these samples were centrifuged (5 min at 2,500 g) and the resulting pellets were washed with distilled water (x2), ethanol (x2) and acetone (x1), with a centrifugation step as indicated above following each washing step. The final pellet, which was free of non-cell wall components and chlorophyll, was dried overnight in an oven (60 ºC) and subsequently weighed. Fiber content of seagrass tissues was obtained from the difference in mass before and after the procedure, and expressed as a percentage of dry biomass.

Natural product analyses

Extraction procedures

Samples (0.5–1.3 g DW) were extracted with MeOH (5mL x 3) under maceration with shaking and sonication for 3min. The resulting slurry was centrifuged for 5 min and the supernatant taken to dryness under vacuum evaporation. The obtained residue (c.a. 50 mg) was partitioned between H_2O and Et_2O, and then n-BuOH (3 x 2 mL). The butanolic extracts were used to analyse the natural products.

UPLC-MS analysis of compounds

Identification of compounds was carried out using an ACQUITY Ultra Performance LC system equipped with a photodioide array detector with a binary solvent system (Waters

corporation, Milford, MA, USA) with a mass detector Xevo G2 Q-TOF mass spectrometer (Waters, Manchester UK) equipped with an electrospray ionization (ESI) source operating in negative mode (Mass Spectrometry Facilities, SC-ICYT, University of Cádiz). Separation of compounds was carried out using a UPLC BEH C18 column (1.7 μm, 2.1 x 50 mm, Waters) at 25 °C. For UPLC-MS analysis, each butanolic extract was suspended in H_2O and a solution of quercetin (Sigma-Aldrich) was added until final concentrations of 1500-2000 μg mL^{-1} for the extract and 2.5 μg mL^{-1} for quercetin were reached (internal standard). The volume of injection was set at 5 μL and the flow rate 0.4 mL min^{-1}. The solvents for the mobile phase were H_2O + 0.1% of formic acid (A) and CH_3CN (B). The following gradient was used: 0.00 min 90% A, 0.00-3.50 min 55.0% A (linear), 3.50-4.20 min 90.0% A (linear), and 4.20-5.00 min 90.0% A (isocratic). The analyses were carried out using a full-scan, data-dependent MS scanning from m/z 100-1000. The mass spectrometer was operated in negative ion mode. The following optimized MS conditions were used: source temperature 120 °C, capillary voltage 3000 V, cone voltage 20V, desolvation temperature 350 °C, and desolvation gas (nitrogen) flow rate 850 L h^{-1}. Characterization of the single components was achieved using the retention times and the accurate molecular masses. Leucine-enkephalin was used as the reference compound. The $[M-H]^-$ ions were detected at 554.2615 Da during an analysis performed within ESI–MS accurate mass experiments, which were permanently introduced via the LockSpray channel using a Hamilton pump. The lock mass correction was ±1.000 for the mass window. Collision-induced fragmentation experiments were performed using argon as the collision gas, with voltage ramping cycles from 0.3 to 2 V. Characterization of the single components was carried out via the retention time and the accurate molecular masses. Each compound was optimized for its estimated molecular mass in the negative mode, before and after fragmentation. The data obtained from UPLC–MS were subsequently entered into MassLynx 4.0ChromaLynx Application Manager software (Waters). For UPLC-MS/MS analysis, the following parameters were used: source temperature 150 °C, capillary voltage 3000 V, cone voltage 20 V, trap collision energy 20-40 eV, and mass scan range m/z 50-1200 Da. The elution of compounds from the column was simultaneously monitored by a photodiode array (PDA) detector at l 300-450 nm.

The quantification of the phenolic compounds (see S1–3 Figures and S2 Table) was performed using external calibration curves of a reference compound selected based on the principle of structure-related target analyte/standard (chemical structure and functional group). The calibration curve for rutin (1) was used to quantify compounds 4 and 6, in addition to being used for its own quantification. The calibration curve for quercetin-3-b-D-glucoside (2) was used to quantify compounds 3, 5 and **7**, in addition to being used for its own quantification. As an internal standard, quercetin was used. All determinations were done in triplicate (n = 3).

Biomechanical analyses

The biomechanical properties of *C. nodosa* leaves were measured with an Instron testing machine (model 5542) and BlueHill® software (v.2.18). We sampled the first outermost fully-developed leaf from the selected EPUs (five leaves per chamber, normally the second youngest leaf), and a portion of the leaf blade was cut 4 to 5 cm above the ligule for testing. The leaves were measured within 1d of sampling and the specimens were tested in the same sequence as they had been collected so that the time of storage was similar among samples and treatments. Leaf-fracture properties were evaluated by two tests (cutting and tearing tests) and expressed at 2 levels: (1) total quantity of force needed to cut or tear a single leaf blade, which depends on the leaf size and its mechanical properties at the material level (F_{TA}, N); (2) material biomechanical traits, normally called 'material properties', which are inherent properties of the material (F_{TS}; N mm^{-2}). Regarding the ecological significance of these traits, whole-leaf biomechanical traits indicate the force needed in absolute terms to cut or tear a single leaf blade by herbivores, whereas material properties show the invested work or force required to ingest an amount of material, giving an idea of the cost-efficiency of the feeding process (De Los Santos et al. 2012). The cutting test measures the force required for foliar breakage (Wright and Vicent 1996; Aranwela et al. 1999). During the test, a force to displacement curve was monitored. Since the whole leaf was cut transversally during the test, the force exerted to cut the lamina also included the leaf veins (De Los Santos et al. 2012). The tensile (tearing) tests were conducted along the long axis of the leaf. The F_{TA} (N) and the F_{TS} (N mm^{-2}) were obtained from the force-displacement curve and the size traits.

Collection of consumers

Sea urchins (*Paracentrotus lividus*) were collected from La Caleta, Cádiz (SW Spain, 36º31'39"N; 6º18'46"W). The most stable populations of *P. lividus* inhabit this place and therefore this area was chosen for the extraction of individuals. After authorization from the local environmental division, 150 individuals of sea urchins were collected from a depth of 2 meters. Harvesting was carefully carried out by snorkelling to avoid damaging the animals. Sizes ranged from 3–5 cm in diameter. Collected individuals were kept in coolers with seawater and transported to the laboratory. Upon arrival, sea urchins were placed in aerated tanks (4 tanks of 30 liters each, 37 individuals in each tank) and were fed with the macroalgae *Ulva* sp. for 3 days until the beginning of the experiment to allow acclimation to laboratory conditions.

Experimental set-up for feeding assays

To experimentally examine how the drivers of change (i.e. elevated temperature, pH level and nutrient input) affect the feeding preference of the sea urchin *Paracentrotus lividus*, we

conducted a suite of no-food choice and food choice feeding assays between *Cymodocea nodosa* from each of the experimental chamber and *Ulva* sp. *Ulva* sp. was used to check the health status of the sea urchins during the different assays (it is voraciously consumed by healthy sea urchins), and as an indicator of the food preference by sea urchins (Boudouresque and Verlaque 2001). Feeding assays were run in a temperature controlled climate room set at 20ºC, where sea urchins were placed in aquaria (individual volume ≈ 20 L) in a closed flow-through system. The aquaria were illuminated by lamps with cool fluorescent tubes (T5 High Output Blau Aquaristic aquarium color extreme fluorescents) in a 8:16h (light:darkness) because *P. lividus* usually exhibits nocturnal activity (Boudouresque and Verlaque 2001). Aeration pits were placed in all aquaria to ensure adequate mixing of water and air. Sea urchins were acclimated for 72 hours prior to starting each assay, during which time they were fed the palatable alga *Ulva* spp. to avoid any interference of previous food or starving on their foraging behaviour.

Two types of feeding assays were conducted: i) individual diet (ID, no food choice assays) and ii) combined diet (CD, food choice assays). In the individual diet assays, we offered 6 g of *C. nodosa* from each of the experimental chambers (8 treatments per 3 replicates, n=24). In the combined diet assays, we offered 3 g of *C. nodosa* from each of the experimental chambers (8 treatments per 3 replicates, n=24) and 3 g of *Ulva sp.*, maintaining the same sea urchin–food ratio as used in the individual diet assays (6 g in total). All feeding assays (n=48) were run with 3 individual sea urchins, which had been starved for 24 h prior to the beginning of the feeding experiment. Each feeding assay was run for 24 h under constant temperature (20ºC) and aeration. We also used control containers during the assays to account for potential changes not resulting from grazing (i.e., seagrass not exposed to herbivores). These did not change in terms of fresh biomass and were thus not considered in the statistical analyses.

After 24 h, the remaining biomass was collected from the tanks, dried and weighed. The results of herbivory were expressed as biomass consumption per individual (i.e. sea urchin) per day. Sea urchins were weighed after each assay to control for their size.

Statistical analyses

Prior to any statistical analysis, data were checked for normality (Shapiro-Wilk normality test) and homoscedasticity (Bartlett test of homogeneity of variances). Treatment differences were analysed using a three-way ANOVA: treatment factors were temperature, pH and NH_4^+ enrichment; response factors were C:N ratio, internal NH_4^+, fiber content, biomechanical properties, natural product content and consumption rate. Consumption rates in the no-choice assays (individual diet) were analysed by means of a three-way ANOVA.

Differences in the two-choice assays (combined diet) between *Ulva* sp. and *C. nodosa* were analysed using a *t*-test (S3 Table). On the other hand, to find differences on *C. nodosa* consumption in the different treatments, a three-way ANOVA (with three fixed factors: temperature, pH and NH_4^+ enrichment) were performed. When significant differences were found, a Tukey post-hoc test was applied.

When statistical differences were not found using the three–way ANOVA, but differences between treatments were large, it may indicate the existence of a power issue because of the limited sample size (n=3), and then a statistical meta-analysis of the effect size was used to avoid the possibly misleading influence of sample size. While null hypothesis significance testing only informs about the probability of an observation, the presentation of the effect size along with its standard error (SE) provides the two most important pieces of statistical information for biologists: the magnitude estimate of an effect of interest and the precision of that estimate (Nakagawa and Cuthill 2007). Thus, if there are non-significant differences but large effects, it may suggest further research with greater power (Fritz et al. 2012). To estimate the effect size of the parameters under study, the Hedges' d metric was chosen (Hedges et al. 1985), as it is an unbiased estimator that provides a better estimate for small sample sizes. The effect size was presented as Hedges' d ± asymptotic standard error for the effect size according to (Nakagawa and Cuthill 2007). Hedges' d metric values above 0 indicate a positive effect, bellow 0 indicate a negative effect, and equal to 0 indicates no effect on the parameter under investigation. The bigger the number either on the positive or negative direction tells about the magnitude of the effect. Cohen, 1988 (Cohen 1988) has proposed 'conventional' values as benchmarks for what are considered to be 'small', 'medium', and 'large' magnitude of the effects (d = 0.2, 0.5, 0.8, respectively) (Nakagawa and Cuthill 2007).

To test for interactions among factors, we used pairwise comparisons when significant interaction terms were detected (Anderson 2006). Data are presented as mean ± s.e. The significance level (α) was set in all tests at 0.05. Pearson's correlation analysis was used to test the possible correlations between the consumption rate of *C. nodosa* in the individual and combined diet assays and the measured properties of *C. nodosa* leaves. Statistical analyses were computed with R 3.0.2 (R Development Core Team 2013).

Results

Leaf structural and nutritional traits

Leaf traits varied significantly among the treatments (Fig 1). Regarding the structural properties of *Cymodocea nodosa* leaves, both elevated temperature and lower pH levels

significantly increased fibre content, with an effect size of about -2.38 and -5.38 for treatments combining ambient and enrichment NH_4^+ concentration respectively, thereby potentially reducing palatability, while combined elevated temperature and NH_4^+ enrichment reduced fibre content (Fig 1), with a negative effect size of about -5.38 and -1.42 combining low and high pH level respectively (Fig 2)

NH_4^+ enrichment also significantly increased leaf thickness ($p<0.01$). In addition, NH_4^+ enrichment positively affected the nutritional quality of *C. nodosa* leaves by decreasing the C:N ratio and increasing internal NH_4^+. In contrast, elevated temperature resulted in the highest C:N ratio (22.2 ± 1.28) as well as the lowest internal NH_4^+ concentration (1.0 ± $2x10^{-5}$ μg NH_4^+ gFW^{-1}), both of which could negatively affect palatability. However, plants subjected to the combination of all three factors (higher temperature, lower pH and higher ammonium) had the highest internal NH_4^+ concentration with a positive effect size value of 1.50.

Natural products

The analyzed samples showed the presence of the following phenolic compounds (see S2): rutin (1), quercetin-3-b-D-glucoside (2), quercetin-3-b-D-glucoside-6´´-acetate (3) isorhamnetin-3-b-rutinoside (4), isorhamnetin-3-b-D-glucoside (5), and two isprenylflavonols (6) and (7). Although compounds 2 and 5 have been previously reported in *C. nodosa* (Cariello et al. 1979), this is the first time that compounds 1, 3, 4, 6 and 7 have been found in this plant, to the best of our knowledge. Leaves from plants acclimated to high temperature contained the highest concentration of phenolic compounds when compared with the rest of treatments ($p<0.05$), with a positive effect size of 3.20 combining low pH level and ambient NH_4^+ concentration. However, when high temperature was combined with lower pH levels, the lowest concentration of phenolic compounds was recorded (Fig 1). Nutrient enrichment, however, did not result in any differences in phenolic compounds among the treatments in both three-way ANOVA test (Fig 1) and effect size analyses (Fig 2).

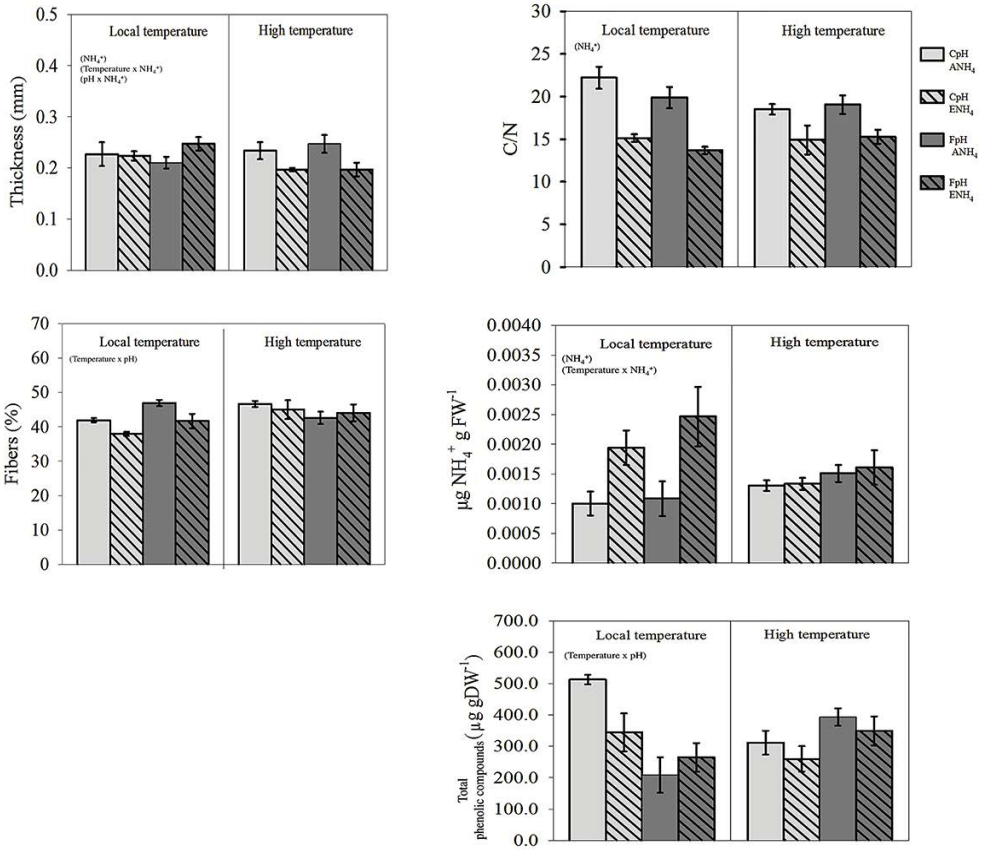

Figure 1. Leaf structural and nutritional traits of *C. nodosa* exposed to warming (local 22 ºC vs high 26 ºC), acidification (current, CpH, vs forecasted, FpH) and eutrophication (ambient ammonium, ANH_4^+, vs enrichment, ENH_4^+). Data are expressed as mean ± s.e. of thickness, fiber content, C:N ratio, concentration of ammonium and concentration of phenolic compounds. Factors resulting in significant differences in the three-way ANOVA are shown in parentheses (p<0.05).

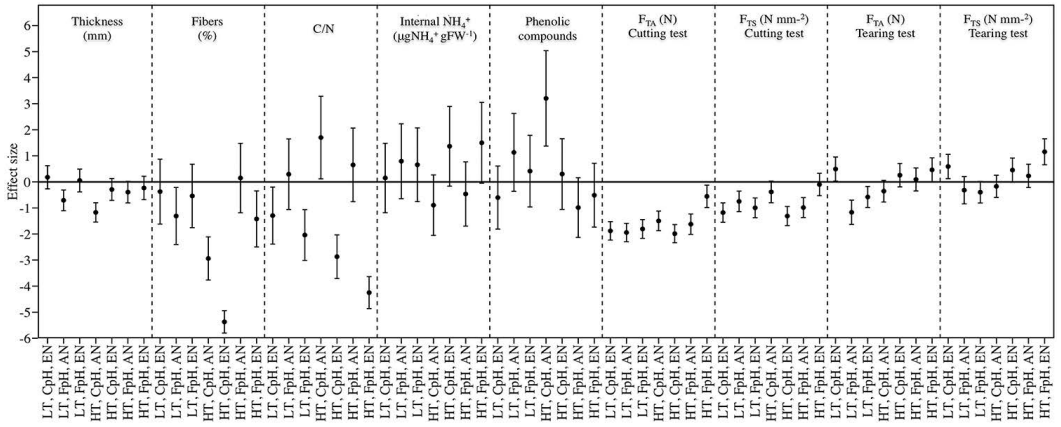

Figure 2. Effect size (n=5) of *Cymodocea nodosa* leaf traits exposed to different temperature (local 22 ºC vs high 26 ºC), pH levels (current, CpH, vs forecasted, FpH) and NH_4^+ concentration (ambient ammonium, ANH_4^+, vs enrichment, ENH_4^+). Error bars indicate the 95% confidence intervals of thickness (mm), fiber content (%), C:N ratio, concentration of internal ammonium ($\mu gNH_4^+ \cdot gFW^{-1}$), concentration of phenolic compounds ($\mu g \cdot gDW^{-1}$, n=3), whole-leaf biomechanical traits (F_{TA}, N) and absolute force-to-tear (F_{TS}, $N \cdot mm^{-2}$) for cutting and tearing test.

Biomechanical traits

Leaves from control plants (local temperature, current pH and ambient NH_4+) were the most resistant (i.e. higher absolute force, F_{TA}) in cutting tests and also had the highest tensile strength (i.e. higher specific force, F_{TS}) (Tables 1 and 2). Both, three-way ANOVA test (Table 2) and effect size analyses (Fig 3) showed two-way interaction effects between lower pH levels and the others factors (increased temperature and NH_4^+ supply), which caused a significant decrease in whole-leaf biomechanical properties (i.e. lower values of F_{TA}, Table 1), and as a consequence, plants showed lower resistance under such factor combinations. However, the three-way interaction between all factors increased the resistance of leaves in comparison to the other interactions, with an effect size of 0.46 for F_{TS} in tearing test (Fig 2). Regarding the tearing test, plants subjected to the combination of the three factors together also showed the most resistant leaves, as well as those with the highest tensile strength.

Table 1. Biomechanical traits of *Cymodocea nodosa* leaves exposed to warming (local 22 ºC vs high 26 ºC), acidification (current, CpH, vs forecasted, FpH) and eutrophication (ambient ammonium, ANH_4^+, vs enrichment, ENH_4^+). Values are means ± s.e. of replicates. For cutting and tearing tests.,Whole-leaf biomechanical traits=F_{TA} (N), absolute force-to-tear; Material biomechanical traits=F_{TS}, specific force-to-tear (N mm^{-2}).

	Cutting test		Tearing test	
Factors	**F_{TA} (N)**	**F_{TS} (N mm^{-2})**	**F_{TA} (N)**	**F_{TS} (N mm^{-2})**
Local T^a, CpH, ANH_4^+	0.564 ± 0.083	0.880 ± 0.12	1.487 ± 0.144	0.995 ± 0.101
Local T^a, CpH, ENH_4^+	0.297 ± 0.077	0.382 ± 0.106	1.693 ± 0.075	1.130 ± 0.113
Local T^a, FpH, ANH_4^+	0.286 ± 0.060	0.533 ± 0.122	1.025 ± 0.105	0.741 ± 0.151
Local T^a, FpH, ENH_4^+	0.309 ± 0.048	0.497 ± 0.077	1.306 ± 0.066	0.825 ± 0.067
High T^a, CpH, ANH_4^+	0.279 ± 0.034	0.417 ± 0.054	1.610± 0.112	1.080 ± 0.081
High T^a, CpH, ENH_4^+	0.360 ± 0.096	0.665 ± 0.152	1.358 ± 0.12	0.892 ± 0.075
High T^a, FpH, ANH_4^+	0.345 ± 0.052	0.501 ± 0.076	1.550 ± 0.124	1.014 ± 0.081
High T^a, FpH, ENH_4^+	0.516 ± 0.113	0.798 ± 0.167	1.813 ± 0.243	1.383± 0.136

Table 2. Results of the three-way ANOVA for biomechanical properties. For abbreviations used in the cutting and tearing tests, see caption for Table 1. Bold font indicates significant differences (p< 0.05).

Cutting test	df	MS	F	p-value
F_{TA} (N)				
Temperature	1	0.224	3.558	0.71
pH	1	0.106	1.687	0.84
NH_4^+	1	0.411	6.529	0.42
Temperature : pH	1	0.637	10.127	**0.035**
Temperature : NH_4^+	1	0.011	0.178	0.11
pH : NH_4^+	1	0.0003	0.005	**0.02**
Temperature : pH : NH_4^+	1	0.03	0.486	0.38
F_{TS} (N mm^{-2})				
Temperature	1	0.291	3.934	0.59
pH	1	0.011	0.152	0.79
NH_4^+	1	0.487	6.587	0.095
Temperature : pH	1	0.939	12.706	0.29
Temperature : NH_4^+	1	0.093	1.269	0.061
pH : NH_4^+	1	0.017	0.231	**0.003**
Temperature : pH : NH_4^+	1	0.052	0.708	0.62
Tearing test				
F_{TA} (N)				
Temperature	1	0.04	0.141	0.064
pH	1	0.01	0.039	0.199
NH_4^+	1	0.20	0.644	**0.013**
Temperature : pH	1	1.46	4.676	**0.0024**
Temperature : NH_4^+	1	0.81	2.589	0.67
pH : NH_4^+	1	1.78	5.69	0.94
Temperature : pH : NH_4^+	1	0.244	0.781	0.48
F_{TS} (N mm^{-2})				
Temperature	1	0.1003	0.298	0.052
pH	1	0.0229	0.068	0.7
NH_4^+	1	0.968	2.876	**0.013**
Temperature : pH	1	0.381	1.132	**0.0008**
Temperature : NH_4^+	1	1.226	3.64	0.26
pH : NH_4^+	1	3.063	9.094	0.63
Temperature : pH : NH_4^+	1	0.085	0.253	0.4

Consumption rate of Cymodocea nodosa

The experimentally manipulated factors (higher temperature, lower pH levels and higher NH_4^+ concentration) increased the consumption rate of sea urchins with respect to the control treatments (Fig 3 and Table 3), both individually and in combination with other factors. In the combined diet treatments, only elevated temperature resulted in significantly increased consumption rates (p<0.05). However, when sea urchins had only seagrass leaves to consume (individual diet), NH_4^+ supply was the variable determining feeding preference, with a positive effect size of 1.75 and 1.96 combining low pH level in high and local temperature treatments (Fig 4). Control plants were consumed at slower rates by sea urchins in both types of diet assays.

In all the feeding assays, *Ulva* sp. was consumed at significant higher rates than the seagrass in both the individual (1.70 ± 0.37 gFW ind^{-1} d^{-1}) and combined diet assays (1.09 ± 0.17 gFW ind^{-1} d^{-1} as a mean of all treatments, S3 Table).

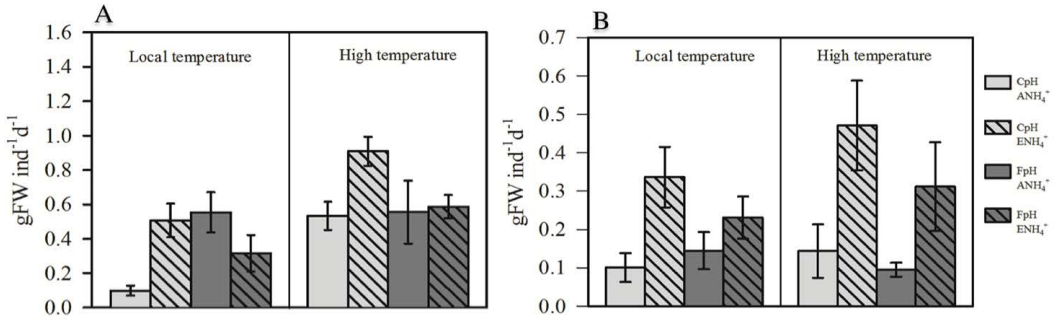

Figure 3. Consumption rates of *C. nodosa* by sea urchins in combined [A] and individual diet treatments [B] (g fresh weight [FW] ind^{-1} d^{-1}; mean ± s.e. *C. nodosa* had been exposed to warming (local temperature vs high temperature), acidification (current pH, CpH, vs forecasted pH, FpH) and eutrophication (ambient ammonium, ANH_4^+, vs enrichment, ENH_4^+).

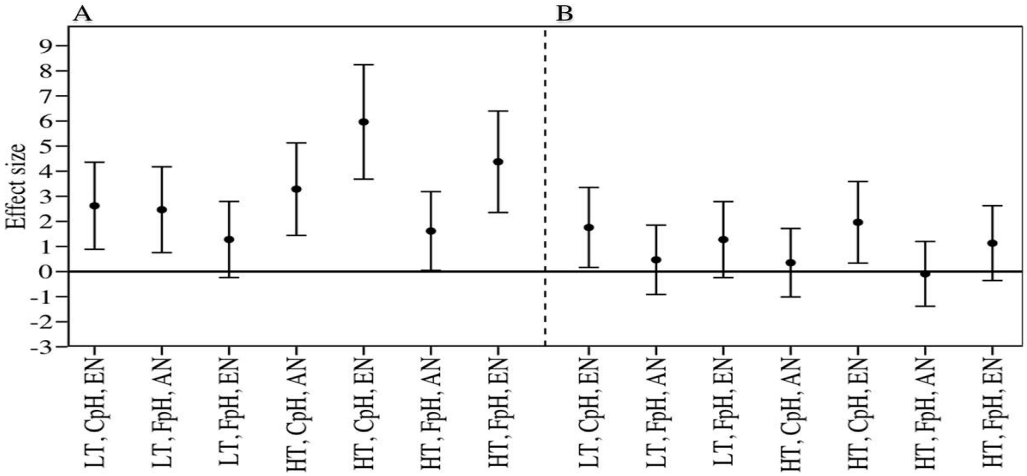

Figure 4. Effect size (n=3) of *Cymodocea nodosa* consumption rate by sea urchins in combined [A] and individual diet treatments [B] (g fresh weight [FW] ind^{-1} d^{-1}. Error bars indicate the 95% confidence intervals. *C. nodosa* had been exposed to different temperature (local temperature vs high temperature), pH levels (current pH, CpH, vs forecasted pH, FpH) and NH_4^+ concentration (ambient ammonium, ANH_4^+, vs enrichment, ENH_4^+).

Table 3. Results of the three-way ANOVA on response of herbivore consumption rate to treatment of *C. nodosa* leaves. Bold numbers indicate significant differences (p< 0.05).

Combined diet				
	Df	**MS**	**F**	**(p-value)**
Temperature	1	0.462	14.18	**0.002**
pH	1	0.0005	0.015	0.9
NH_4^+	1	0.125	3.846	0.68
Temperature : pH	1	0.119	3.654	0.07
Temperature : NH_4^+	1	0.021	0.649	0.43
pH : NH_4^+	**1**	0.368	11.297	**0.004**
Temperature : pH : NH_4^+	1	0.034	1.054	0.32
Individual diet				
	Df	**MS**	**F**	**(p-value)**
Temperature	1	0.016	0.955	0.34
pH	1	0.027	1.602	0.22
NH_4^+	1	0.28	16.529	**0.0009**
Temperature : pH	1	0.008	0.472	0.5
Temperature : NH_4^+	1	0.018	1.093	0.31
pH : NH_4^+	1	0.025	1.494	0.24
Temperature : pH : NH_4^+	1	0.0005	0.034	0.85

Correlation of consumption rates and leaf traits

In the pairwise comparisons between leaf traits (i.e. structural, nutritional, biomechanical and chemical) and consumption rates (for individual and combined diets), we found a significant positive correlation between leaf N content and consumption rate in the individual assays ($p<0.05$; Table 4). Also, a negatively correlation between fiber content and consumption rate was found in the combined diet assays ($p<0.05$). In both feeding assays (individual and combined diet), FTS (in cutting tests) was negatively correlated to consumption rates ($p<0.05$).

Table 4. Pairwise Pearson's correlation coefficients between herbivore consumption rate and structural, nutritional, biomechanical and chemical traits of leaves. Correlations between averaged values for each treatment (n=3). Statistical significance indicated by bold font (p<0.05). Structural traits: C, leaf carbon content; NDF, leaf fibre content; Th, leaf thickness. Nutritional leaf traits: N, leaf nitrogen content; internal NH_4^+, internal ammonium. Whole-leaf biomechanical traits: F_{TA}, absolute force-to-tear (N). Material leaf biomechanical traits: F_{TS}, specific force-to-tear (N mm^{-2}). Chemical traits: Phenolic compounds (µg gDW^{-1}).

		Structural traits			**Nutritional traits**		**Cutting test**		**Tearing test**		**Chemical traits**
		C	**NDF**	**Th**	**N**	**NH_4^+ internal**	**F_{TA}**	**F_{TS}**	**F_{TA}**	**F_{TS}**	**Phenolic compounds**
Combined diet	Correlation	-0.28	-0.49	-0.19	0.16	0.27	-0.24	-0.54	0.29	0.22	-0.26
	p-value	0.19	**0.015**	0.37	0.44	0.19	0.24	**0.006**	0.17	0.3	0.26
Individual diet	Correlation	-0.21	-0.29	-0.11	0.39	0.3	-0.2	-0.54	0.27	0.13	0.06
	p-value	0.33	0.16	0.59	**0.04**	0.15	0.34	**0.006**	0.207	0.55	0.79

Discussion

The assayed factors temperature (local and elevated temperature, 22ºC *vs* 26ºC), pH levels (current and forecasted pH, 8.12 vs 7.69) and NH_4^+ concentration (ambient and enriched NH_4^+ concentration, 0 and 31 μM) , acting independently or in combination, modified the structural, nutritional, biomechanical and chemical properties of *Cymodocea nodosa* leaves, thereby affecting their palatability. Plant acclimated to NH_4^+ enrichment alone and in combination with the other two factors showed simultaneously higher N content, internal NH_4^+ content in leaves and reduced resistance of the leaves (lower F_{TA}) (Fig 1). Moreover, lower pH levels decreased the resistance of leaves and concentration of phenolic compounds (Fig 1, 2 and Table 2). As a consequence, under such conditions, leaf tissues may be more palatable and, therefore, more vulnerable to consumption since herbivores typically adjust their consumption rates relative to the nutritional content of their food source (Cebrián et al. 2009). Thus, we found in both types of feeding assays that the consumption rates of *C. nodosa* leaves acclimated to predicted future levels of the three factors (independently or in combination) was higher than those recorded for control plants, in both three-ways ANOVA (Fig 3) and effect size analyses (Fig 4). Therefore, it is expected that both global (i.e. increase in temperature and acidification) and local shifts (i.e. eutrophication) will trigger an increase in the grazing of seagrass tissues.

Several studies have demonstrated a direct relationship between leaf nitrogen content and grazing (McGlathery 1995; Goecker et al. 2005). However, others authors have found opposite patterns (Mariani and Alcoverro 1999; Kirsch et al. 2002; White et al. 2011). This suggests that the decision by herbivores to feed on one species or another does not depend only on one factor but may depend on a combination of factors. Here, we present the first study that analyzes a broad spectrum of leaf properties that define plant palatability under the main drivers of change in seagrass ecosystems (warming, acidification and eutrophication) (Waycott et al. 2009). First, we showed that NH_4^+ enrichment increased nutritional quality (i.e. higher N content and lower C:N ratios) and enhanced seagrass susceptibility to consumption by sea urchins, showing a positive correlation with N content in leaves in accordance with general predictions (Valentine and Duffy 2006; Cebrián et al. 2009). We also recorded negative correlations between consumption rate and responses expected to decrease grazing (Vergés et al. 2008), such as structural (i.e. thickness, fiber and carbon content), chemical (i.e. phenolic compounds) and biomechanical defense traits (resistance of leaves) (Table 4). Some studies analysing plant traits that mediate feeding choices of seagrasses by consumers have observed structural traits (Prado and Heck 2011; Vergés et al. 2011; Tomas et al. 2015) and chemical compounds acting to deter feeding on seagrass leaves (Vergés et al. 2007). Others have hypothesized that biomechanical traits [41] may affect the quality of a plant as suitable food, with increased tissue resistance making leaves less palatable because of the greater effort

needed to tear off and chew tissue. Here, we present experimental findings on changes in biomechanical properties under a global change scenario, their relations to other leaf properties and their influence on feeding decisions by a generalist herbivore. We observed a reduction in leaf resistance (lower F_{TS} and F_{TA} in cutting test analyses) under the acclimation of different global factors such as the combination of high temperature and low pH levels or local factors such as NH_4^+ enrichment. Therefore, these results in combination with previous findings demonstrate how nutrient enrichment is the main driver of seagrass consumption by changing not only nutritional quality (Cebrián et al. 2009) but also biomechanical traits, enhancing seagrass susceptibility to consumption.

However, NH_4^+ enrichment was not the only trigger for changes in foliar properties and, thus, the consumption of plants. High temperature in combination with the other two factors (i.e. low pH and nutrient enrichment) acted to reduce the resistance of leaves as well as lowering C:N ratios, thickness and phenolic compounds, making plants more palatable to herbivores. Accordingly, studies have found that warming temperatures increased plant susceptibility to herbivores (e.g., C:N ratio (Staehr and Wernberg 2009) but also increased secondary defense metabolites (Sudatti et al. 2011). Others have shown a reduction of secondary metabolites under the scenario of ocean acidification, in *C. nodosa* leaves and *Posidonia oceanica* seedlings (Arnold et al. 2012; Hernán et al. 2016), suggesting enhanced susceptibility of seagrass to grazing pressure. A notable point of this work is the identification of phenolic compounds not previously reported for *C. nodosa (*see S2 Fig*)*. Among the several metabolites that plants produce, we focused attention in the phenolic compounds in particular because they bear a variety of deterrent and ecological functions (Arnold and Targett 2002). These compounds, characterised by a carbon-based structure, with one or more hydroxyl groups bounded to an aromatic ring, have been the main products found in *C. nodosa* (e.g. caffeic acids and flavonoid glucosides such as quercetine and isorhamnetine monoglucosides*,*(Cariello et al. 1979; Sica et al. 1984). Moreover, it is known that phenolic acids and condensed tannins are commonly used by marine macroalgae to reduce palatability or increase toxicity to herbivores (Steinberg 1985; Steinberg et al. 1991; Targett et al. 1995; Pavia and Toth 2000; Arnold and Targett 2002; Jormalainen and Honkanen 2008). Interestingly, past studies have observed that low pH conditions could alter the concentration of these compounds in *C. nodosa*, reporting a loss of simple and polymeric phenolics in their leaves (Arnold et al. 2012). While we also detected a decrease in leaf phenolic content under low pH levels, an increase was actually obtained when combined with high temperature. This combination also increased fiber content but reduced leaf resistance, resulting in a net balance between leaf traits that increase susceptibility to grazing pressure and those that reduce it. Hence, we can conclude from this study that leaf trait responses can be highly variable, depending on whether driving environmental changes are considered as a single factor or in

combination, and this will affect predictions of how these drivers will influence herbivory in ecosystems in the future.

Conclusion

In summary, the study shows how the main factors of globally and locally driven change in seagrass communities (warming, acidification and eutrophication) modify a broad spectrum of leaf traits, including the first set of empirical data on biomechanical traits. These changes led to an overall increase in plant palatability and, therefore, enhanced their consumption by a generalist herbivore *Paracentrotus lividus*, which could strengthen top-down effects in seagrass habitats and modify the fluxes of matter and energy in coastal areas. (Lavery et al. 2013). Predicting the net outcome of these co-occurring disturbances still remains a pressing challenge (Crain et al. 2008), and our findings suggest that more studies are needed to understand the direct and indirect impacts caused by change driven at the global and local scales and their effects on herbivory in seagrass communities.

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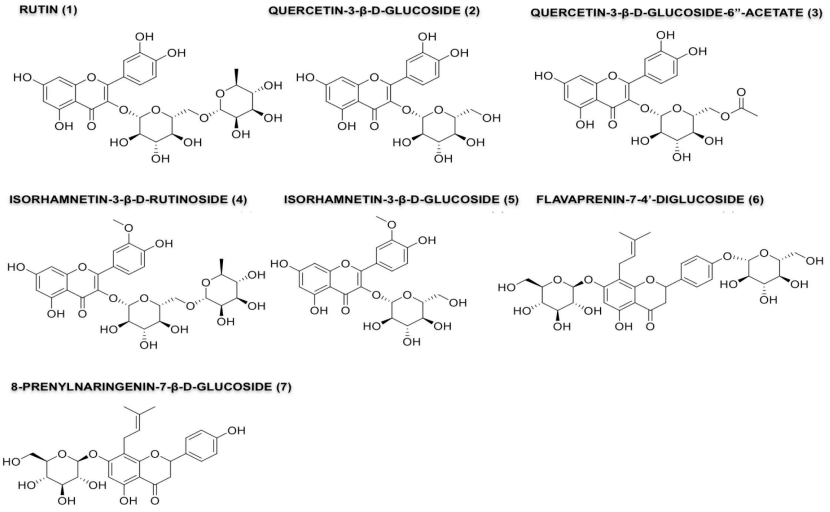
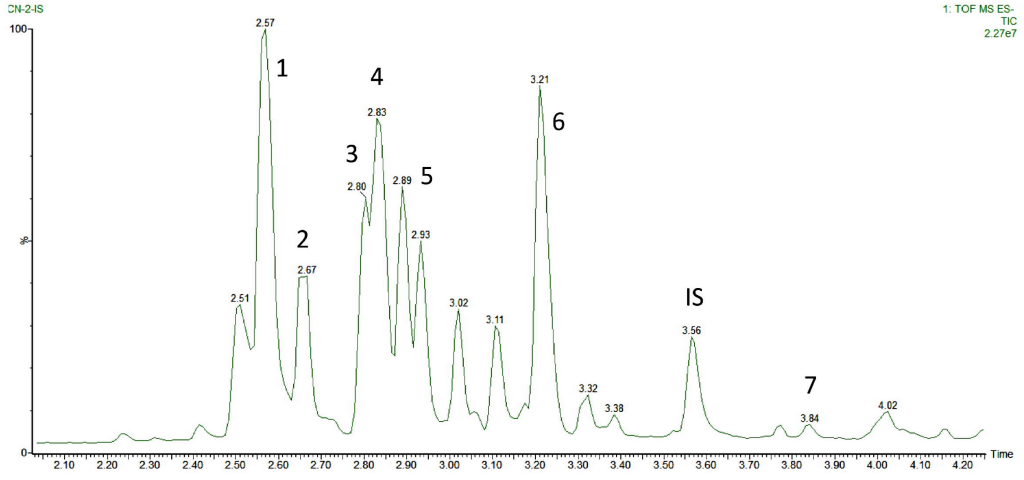
Supporting Information

S1 Table. Control measures for each treatment. Data are means ± s.e. CpH: Current pH; FpH: Forecasted pH; ANH_4^+: Ambient NH_4^+; ENH_4^+: Enrichment NH_4^+.

Incubation treatments			NH_4^+ (µM) *	pH	pCO_2 (ppm)	Temp. (°C)	DIC (µmol kg $^{-1}$)
T[a]	pH	NH_4^+					
Local	CpH	ANH_4^+	0	8.14 ± 0.01	447 ± 21	21.93 ± 0.08	2358 ± 30
Local	CpH	ENH_4^+	30.4 ± 1.3	8.13 ± 0.02	424 ± 22	21.94 ± 0.08	2179 ± 17
Local	FpH	ANH_4^+	0	7.66 ± 0.01	744 ± 17	21.85 ± 0.05	1275 ± 22
Local	FpH	ENH_4^+	31.8 ± 1.7	7.68 ± 0.01	750 ± 18	21.95 ± 0.08	1287 ± 30
High	CpH	ANH_4^+	0	8.10 ± 0.01	412 ± 18	26.08 ± 0.02	2278 ± 23
High	CpH	ENH_4^+	31.4 ± 1.5	8.10 ± 0.02	402 ± 19	26.07 ± 0.03	2214 ± 12
High	FpH	ANH_4^+	0	7.66 ± 0.01	736 ± 12	26.10 ± 0.03	1210 ± 49
High	FpH	ENH_4^+	32.1 ± 1.4	7.67 ± 0.01	729 ± 12	26.14 ± 0.03	1238 ± 15



S1 Figure. Chemical structures of compounds 1-7.



S2 Figure. Representative UPLC-MS chromatogram and selected MS/MS data of the detected compounds. Total ion current chromatogram obtained by UPLC-MS for a general extract obtained from *Cymodocea nodosa*. Numbers indicate the compounds detected. IS: internal standard used for quantification.

S2 Table. Identification of phenolic compounds by UPLC-ESI-MS in *C. nodosa.*

Comp.	t_R (min)	Formula	MS² focused on [M-H]⁻ (m/z)*	Identification
1	2.57	$C_{27}H_{29}O_{16}$	609.1437 [M-H]⁻ (18), 301.0329 [M-H-308]⁻ (100)	**
2	2.67	$C_{21}H_{19}O_{12}$	463.0846 [M-H]⁻ (20), 301.0328 [M-H-162]⁻ (100)	**
3	2.80	$C_{23}H_{21}O_{13}$	505.0963 [M-H]⁻ (25), 463.0845 [M-H-42] ⁻ (10), 301.0325 [M-H-42-162]⁻ (100)	ref. 1
4	2.83	$C_{28}H_{31}O_{16}$	623.1577 [M-H]⁻ (35), 315.0502 [M-H-308]⁻ (100)	ref. 2
5	2.89	$C_{22}H_{21}O_{12}$	477.1010 [M-H]⁻ (15), 315.0510 [M-H-162]⁻ (100)	ref. 1
6	3.21	$C_{26}H_{29}O_{10}$	501.1773 [M-H]⁻ (38), 339.1230 [M-H-162]⁻ (100), 219.0645 [M-H-162-120]⁻ (30)	ref. 3, 4
7	3.84	$C_{26}H_{29}O_{10}$	663.2286 [M-H]⁻ (20), 501.1765 [M-H-162]⁻ (35), 339.1211 [M-H-162-162]⁻ (100), 219.0645 [M-H-162-162-120]⁻ (58)	ref. 3,5

*values in brackets represent relative abundance **standard available.

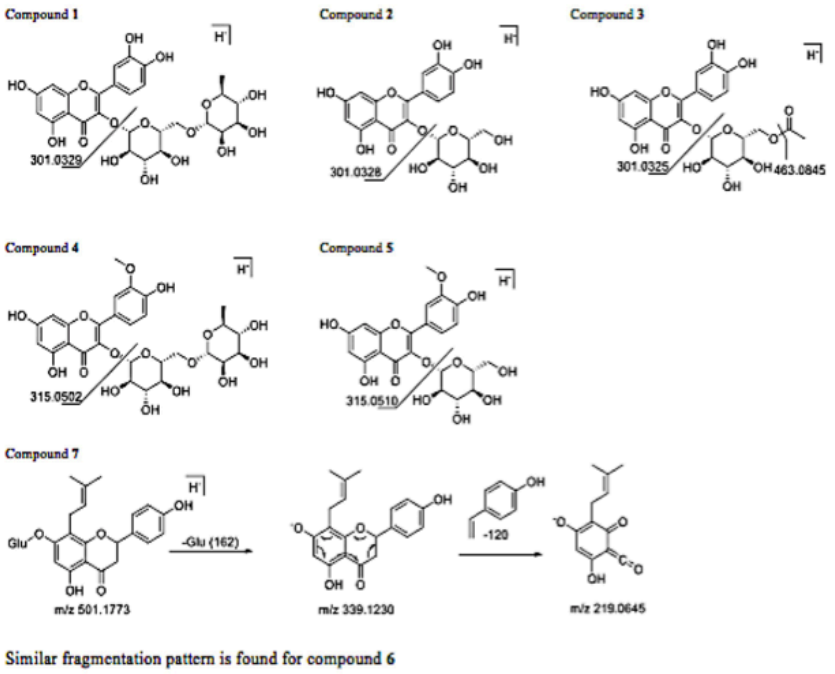
Ref.1 a) Ma, Y., N. Tanaka, A. Vaniya, T. Kind, O. Fie. 2016. Ultrafast Polyphenol Metabolomics of Red Wines Using MicroLC-MS/MS. J. Agric. Food Chem. **64**: 505-512. b) Lin, L.Z., J. Sun, P. Chen, J. Harnly. 2011. UHPLC-PDA-ESI/HRMS/MS^n Analysis of Anthocyanins, Flavonol Glycosides, and Hydroxycinnamic Acid Derivatives in Red Mustard Greens (*Brassica juncea* Coss Variety) . Agric. Food Chem. **59**: 12059-12072.

Ref. 2 Mokrani, A., S. Krisa, S. Cluzet, G. Da Costa, H. Temsamani, E. Renouf, J.M. Mérillon, K. Madani, M. Mesnil, A. Monvoisin. 2016. Phenolic contents and bioactive potential of peach fruit extracts. Food Chem. **202,** 212-220.

Ref. 3 Quifer-Rada, P., A. Vallverdú-Queralt, M. Martínez-Huélamo, G. Chiva-Blanch, O. Jauregui, R. Estruch, R.A. Lamuela-Raventós. 2015. Comprehensive characterisation of beer polyphenols by high resolution mass spectrometry (LC-ESI-LTQ-Orbitrap-MS). Food Chem. **169**: 336-343.

Ref. 4 Kim, H.Y., S.H. Kim, B.Y. Kang, I.S. Lee. 2008. Neuroprotective effects of medicinal plants Arch. Pharm. Res **31**: 1241-1246.

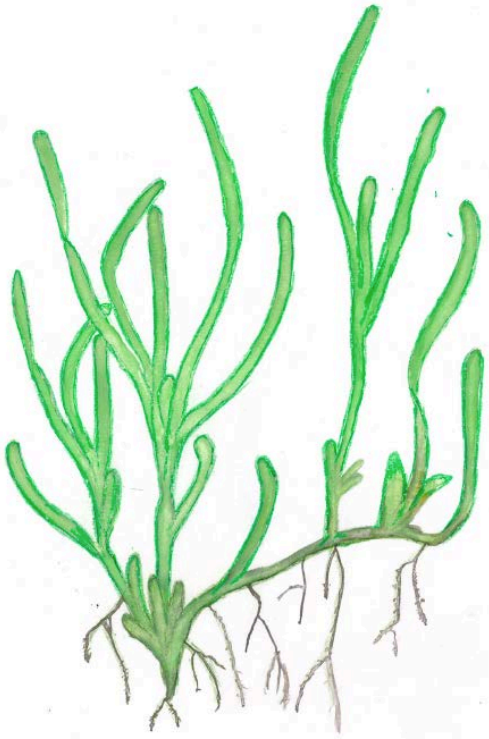
Ref. 5 Arisawa, M., T. Horiuchi, T. Hayashi, Y. Tezuka, T. Kikuchi, N. Morita. 1993. Studies on constituents of *Evodia rutaecarpa* (Rutaceae). I. Constituents of the leaves. Chem. Pharm. Bull. **41**: 1472-1474.



S3 Figure. Main MS fragmentation of identified compounds.

S3 Table. The statistics obtained from paired *t*-tests in consumption rate between *Ulva* sp. and *C. nodosa* in combined diet assays. Bold letters indicate significant differences.

	t	df	*p*-value
Local T[a], CpH, ANH_4^+	-7.568	4	**0.0016**
Local T[a], CpH, ENH_4^+	-3.978	4	**0.016**
Local T[a], FpH, ANH_4^+	-6.173	4	**0.0034**
Local T[a], FpH, ENH_4^+	-4.594	4	**0.01**
High T[a], CpH, ANH_4^+	-3.088	4	**0.036**
High T[a], CpH, ENH_4^+	-1.644	4	**0.017**
High T[a], FpH, ANH_4^+	-2.971	4	**0.041**
High T[a], FpH, ENH_4^+	-4.41	4	**0.011**



CHAPTER 6

Differential responses to simulated grazing in *Halodule wrightii* patches of different colonization states

In preparation

Sueña y serás libre de espíritu. Lucha y serás libre en vida.
Dream and you will be free in spirit. Fight and you will be free in life.

El Che

Differential responses to simulated grazing in *Halodule wrightii* patches of different colonization states

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Abstract

Climatological disturbances can generate heterogeneous environments and profoundly influence plant community by creating patches at different successional stages. Herbivores, in turn, can govern plant succession dynamics by determining the rate of species replacement, ultimately affecting plant community structure. Plants have evolved strategies to counteract or mitigate such negative effects to herbivore attacks being crucial process to better understand ecological and evolutionary processes. However, little is known about such strategies in seagrass communities. In this context, our work was aimed to study the tolerance and resistance strategies to herbivory damage in the tropical seagrass *Halodule wrightii,* identifying specific responses in three patches with different colonization states (patches from 2010, 2012 and 2014). Our approach was based on a field experiment consisting of clipping the seagrass leaves and measuring subsequent plant responses in terms of biomass changes, leaf growth, carbon and nitrogen contents in tissues and total non-structural carbohydrates. *H. wrightii* exhibited differential plasticity in response to herbivore pressure depending on colonization states. Old patches induced substantial compensatory responses, such as enhanced growth through mobilisation of internal reserves. In contrast, in young patches there was no trade-off between compensatory growth and cost for internal reserves. These results demonstrate that herbivores can have an important role in seagrass community structure and function, and can be a key force during succession dynamic.

Introduction

Herbivory is considered as a key ecological process that determines the fluxes of energy and matter from primary producers to higher trophic levels (McNaughton et al., 1989; Hulme 1996). The long evolutionary history of plant-herbivore interactions is reflected in the large amount of adaptive mechanisms and strategies displayed by plants to avoid consumption by herbivores or to mitigate its consequences (e.g. Karban and Baldwin, 1997; Bingham and Agrawal, 2010). Plant defenses against herbivory have been categorised into three main strategies: escape from consumers, resistance against herbivores or tolerance to herbivory (Luchenco and Gaines, 1981; Karban and Baldwin, 1997; Strauss and Agrawal 1999). Resistance strategies are based on nutritional, chemical, or structural plant adaptations that lower plant quality and palatability and reduce herbivore preference or performance (Fritz and Simms 1992). On the other hand, tolerance refers to the ability of plants to withstand and survive herbivory, and it is a property of genotypes defined as the extent to which plant fitness is affected by tissue damage relative to fitness in the undamaged state (Stowe et al. 2000). Both tolerance and resistance strategies entail costs and benefits, and are not considered mutually exclusive (Mauricio et al 1997). Past studies have described a suite of tolerance responses following natural or simulated herbivory, including compensatory growth, increased photosynthetic rate or changes in nutrients allocation pattern (Strauss and Agrawal, 1999). Meanwhile, resistance strategies have been often achieved through secondary metabolites production (Wu and Baldwin, 2010), or by changes in tissue toughness or in other mechanical properties (Lucas et al., 2010).

Herbivory have been postulated to be greater in marine than in terrestrial systems (Cry and Pace, 1993). In fact, plant biomass lost through herbivory is 5-10 times higher in marine systems (Bakker et al., 2016). Especially, coastal seagrass communities are the basis of many coastal food webs (Preen, 1995; Prado et al., 2007; Fourqurean et al., 2010) and share some similar traits with terrestrial plants consistent with adaptations to grazing such as basal meristems or belowground storage (Hek and Valentine, 2006; Marbà et al., 2006; Valentine and Duffy, 2006). However, plant defense strategies have been less explored in seagrasses than in terrestrial plants. There are evidences that seagrasses can tolerate grazing by means of both intensifying recruitment of new shoots (Valentine et al., 1997) and compensatory growth (Valentine et al. 1997; Heck et al. 2000; Vergés et al. 2008; Sanmartí et al. 2014). Indeed, many experiments reported an increase in leaf growth as a compensatory responses after leaf loss by herbivory in seagrasses (Vergés et al., 2008; Sanmanrtí et al., 2014) which can be coupled to the utilization of stored reserves (Sanmartí et al., 2014). On the other hand, the resistance strategies to herbivory attack remain in general poorly known (Vergés et al., 2007; Steele and Valentine 2012).

Seagrasses form an unique group of flowering plants that have adapted to live fully submersed in the sea, providing a large set of ecological services to both coastal areas and citizens (den Hartog 1970; Short and Wyllie-Echeverria 1996; Hemminga and Duarte 2000). In spite of their ecological and economical importance, a worldwide decline has been recorded in the last decades in these communities because of anthropogenic and natural causes (Waycott, 2009). Besides coastal ecosystem disturbances from human activities, the majority of seagrass meadows found in tropical areas (Green & Short 2004) are regularly exposed to flooding, cyclones or hurricanes, which negatively impact seagrasses either physically (via burial, scouring and direct removal of plants and seed banks; Preen & Marsh 1995, Bach et al. 1998, Campbell & McKenzie 2004) or physiologically (via light limitation, nutrient excess and low salinity; Bjork et al. 1999, Ralph et al. 2007). Moreover, other natural disturbance such El Niño, can change the diets of tropical seagrass consumers (Mazariegos-Villarreal et al. 2012). The frequency and intensity of these extreme climatological events are expected to increase as a consequence of climate change (e.g. Duarte et al. 2006) and then, its effects in seagrass beds may be higher, causing great impacts and mortality. The recovery of seagrass beds after extreme climatological events is slow (Reynolds et al. 2013), and uses to take up to 4 years (Preen 1995), although longer periods were also recorded (Bulthuis and Woelkerling 1981; Birch and Birch 1984; Onuf 2000; Blake and Ball 2001). Studies on seagrass colonization (e.g. Duarte and Sand-Jensen, 1990; Barrón et al. 2004) and recovery (van Katwijk et al. 2016) showed that relatively young seagrass meadows have different above and belowground structural complexity, fauna assemblages and sediment stabilization properties when compared to mature seagrass meadows. Therefore, disturbances can generate heterogeneous environments and profoundly influence plant community by creating patches at different successional stages. Herbivores, in turn, can govern plant succession dynamics by determining the rate of species replacement, ultimately affecting plant community structure (Aquilino and Stachowicz 2012, Daleo et al. 2014).

In this context, we experimentally evaluated the role of herbivory in the recovery following climatological disturbance of the seagrass community and assessed whether herbivory affects to the response mechanisms of the tropical seagrass *Halodule wrightii*, a relatively rapid colonisers with fast clonal growth rates (Rasheed 2004). Our approach was based on an *in situ* experiment consisting of clipping the seagrass leaves and measuring subsequent plant responses in terms of biomass changes, leaf growth, carbon and nitrogen contents in tissues and total non-structural carbohydrates in seagrass patches with different recovery from disturbance.

Material and Methods

Study site

Caleta Balandra is a protected natural area and a Natural Heritage by the UNESCO since 2013 located in Bahía de La Paz (southern Gulf of California, Mexico) (Figure1). The area contains a landscape mosaic composed by mangroves, a coastal lagoon, rocky reef areas and monospecific patches of the seagrass *Halodule wrightii* Ascherson. *Halodule wrightii* is commonly distributed in subtropical regions of Caribbean and Gulf of Mexico coasts and contributes to a significant percentage of benthic primary production in these areas (Virnstein 1982; Dunton and Tomasko 1994; Gallegos et al. 1994; Tomasko and Dunton 1995). Furthermore, this species is considered as a pioneering species, and therefore a fast growing one responding quickly to external stressors (Unsworth et al. 2015).

The system is linked to strong tidal currents and sediment fluxes, which intensifies during hurricanes season (from summer to winter), leading to changes in the distribution of *H. wrightii* patches. As a consequence, the area bears a variety of patches with different succession dynamics because of the natural perturbation they support (Pérez-Estrada, in prep, monitoring from 2010 to now). In fact, Pérez-Estrada (in prep.) found a large variety of patches with different colonization states since an extreme climatological event (tropical storm), which buried and/or removed completely the seagrass meadow, in a monitoring work starting in 2010.

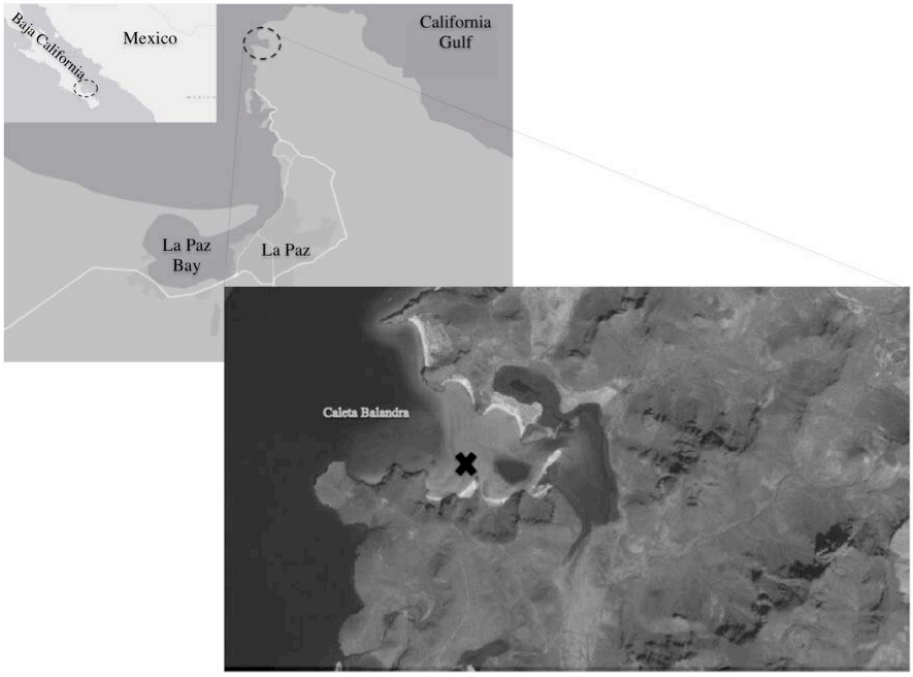

Figure 1. Map of the study area in Caleta Balandra (Baja California Sur, Mexico) showing the location (dot) of *Halodule wrightii* patches where the *in situ* experimental plots were deployed.

The study was conducted in a 4 m deep (high tide) *H. wrightii* patches (24°19'N 110°20'W) with 3 different colonization states (i.e. three different colonization states meadow since patch was re-established): i) from 2010 (old patches), ii) from 2012 (medium patches) iii) from 2014 (young patches). The different patches were spaced at least 10 meters far away each other.

Experimental design

We simulated grazing pressure by herbivores by clipping the leaves of *H. wrightii* during spring 2015. This period was chosen because it is the previous period to hurricanes season. The leaf clipping tried to mimic the aboveground removal done by herbivores, which only consume the leaves of a single shoot at every feeding attack and which do not feed over the same shoots selectively (i.e. macroherbivores, fishes or equinoderms).

We established 6 plots of 20x20 cm in each patch. Two treatments, i.e. control (n=3) and clipped (n=3), were randomly assigned to each plot. In the clipped treatment the leaf canopy was cut to 2 cm height above sediment level (which corresponds to removal of about

50% of leaf biomass approximately). All clipped blades were removed from the plots to avoid any artefact derived from detritus accumulation. In the control plots, the leaf canopy was left un-modified, thus remaining at its normal height (ca. 4.5 ± 0.8 cm above the sediment level for each patches). The apex of control shoots was examined at the end of the experimental period and no marks of recent grazing were observed.

Leaf growth was measured in control and clipped plots using a modified Zieman method (Peralta, 2000) in five shoots with sufficient leaves width (1–2 mm) to employ the hole-punching method (Hauxwell et al. 2001). Selected shoots were carefully marked in each plot by punching a hole just above the ligule of the outermost leaf using a hypodermic needle. All marked shoots were collected at the end of the experiment. At the end of experimental period (1 month) samples of different treatments of *H. wrightii* patches were collected using a 20x20 cm diameter corer. All rhizomes, roots, leaves and sediment were removed and placed in a plastic bag. In the field and during transportation, all material was kept in an ice chest until stored in -24°C freezer before being processed in the laboratory. Once in the laboratory, several plant response variables (biomass, growth, fibre content, C:N and non-structural carbohydrates) were measured in sampled plants.

Measurement of plant response variables

a) Physiological analyses

At the end of the experimental period (1 month), samples of different treatments of *H. wrightii* patches were collected using a 20 cm diameter corer. All rhizomes, roots, leaves and sediment were removed and placed in a plastic bag. In the field and during transportation, all material was kept in an ice chest until stored in -24°C freezer before being processed in the laboratory.

Above- and belowground plant material was separated into leaves, roots and rhizomes, counted, weighed and dried for 3 days at 60°C to obtain dry weight (DW). Biomass was expressed as g DW m^{-2}. Shoot density was determined from the total number of shoots within the area collected and expressed as shoot m^{-2} (n=3 per treatment). Subsamples of each fraction were used for biochemical analysis. The leaves from each punched shoots were separated and number of leaves and length of each leaf was recorded. Leaves were dried at 60°C for 48 h (until constant weight) and weighed.

Moreover, leaf growth rates, leaf elongation rate and leaf renovation rates were calculated for control and clipped leaves from the three patches:

i. Leaf growth rates (LGR) expressed as cm ·d^{-1}leaf^{-1} were calculated by: LGR= (TLL_e-TLL_i)/t, where TLLe is the total leaves length (cm) (i.e. sum of the all leaves length per shoot) at the end of the experiment and TLLi is the total leaves length at the initial time of the experiment and t is the experimental time (30 days). Leaf gross growth rate was calculated by dividing the distance travelled by holes and number of days (cm ·d^{-1}).

ii. Leaf elongation rate (LER; cm shoot^{-1} d^{-1}) was calculated by dividing the length of new tissue produced by the number of days elapsed since marking (i.e. $LER = \frac{\sum_{i=1}^{n}[(LLe-LLi)>0]}{t}$, where LL is the leaf selected length (cm) and n the number of leaves at end of the studied period (t=30 days).

iii. Leaf renovation rates (LRR; d^{-1}) were estimated in length changes, by dividing the leaf elongation rate and total length of leaves at initial time (t=0).

b) Tissue biochemical analysis

In each plot biochemical analyses were conducted in above and below biomasses of *H. wrightii*, in 5 independent samples. Prior to measurements, seagrass leaves were cleaned of epiphytes carefully using a piece of soft paper. The water content in the samples was calculated as the weigh difference between fresh and dried biomass after 48 hours in the oven (60ºC). Subsamples of each plot sample were dried to 60º during 48h to determine the total C and N content in tissues and fibre content. Elemental analysis was performed in a Perkin-Elmer 2400 elemental analyzer, while fibre content was done using the method of (Van Soest et al. 1991) modified by de los Santos (2012).

For carbohydrates, the concentrations of sucrose and starch were measured on duplicate leaf and rhizome samples from each plot. Total non-structural carbohydrates (TNC) were measured following Alcoverro et al. (1999) and Brun et al. (2002). Sugars (sucrose and hexoses) were first solubilized by 4 sequential extractions in 96% (v/v) ethanol at 80°C for 15 min. The ethanol extracts were evaporated under a stream of air at 40°C and the residues were then dissolved in 10 ml of deionized water for analysis. Starch was extracted from the ethanol-insoluble residue by keeping it for 24 h in 1 N NaOH. The sucrose and starch content of the extracts was determined spectrophotometrically using a resorcinol and anthrone assay with an absorbance of 486 and 640 nm, respectively, with sucrose as a standard.

Statistical analyses

A two-way ANOVA and a Tukey's post hoc analyses were applied to assess significant differences in tolerance and resistance strategies (i.e. changes biomass, leaf growth,

leaf elongation rate, leaf renovation rate, shoot density, carbon and nitrogen content in tissues and total non-structural carbohydrates). Data were transformed (i.e. starch in above biomass) when necessary to meet ANOVA assumptions of normality (Shapiro-Wilks test) and homogeneity of variances (Barlett test). Moreover, when ANOVA assumptions were not satisfied (i.e. leaf growth, leaf elongation rate, leaf renovation rate, C:N below biomass and sucrose in above biomass) a non-parametric comparison (Kruskal Wallis matched pairs test) was applied to assess statistical differences. The significance level was set at 0.05 of probability.

A meta-analysis of effect size to avoid the possibly misleading influences of sample size was made in order to determinate the difference in seagrass responses to simulated herbivory. Effect size and its confidence interval enable to make more biologically relevant decisions because allows effective statistical inference from data, offering a better understanding and characterization of the results (Nakagawa and Cuthill 2007). While null hypothesis significance testing only informs about the probability of an observation, the presentation of effect size along with its standard error (se) provide the two most important pieces of statistical information for biologists: the magnitude estimate of an effect of interest and the precision of that estimate (Nakagawa and Cuthill 2007). Thus, if there are non-significant differences but large effects, it may suggest further research with greater power (Fritz et al. 2012). To estimate the effect size of the parameters under study we chose the Hedges' d metric (Hedges et al. 1985) because it is an unbiased estimator that provides a better estimate for small sample sizes. Effect size is present as Hedges' d ± standard error (se). Hedges' d metric values above 0 indicate a positive effect, bellow 0 indicate a negative effect, and equal to 0 indicates no effect on the parameter under investigation. The bigger the number either on the positive or negative direction tells about the magnitude of the effect. Cohen (1988) has proposed 'conventional' values as benchmarks for what are considered to be 'small', 'medium', and 'large' magnitude of the effects (d = 0.2, 0.5, 0.8, respectively) (Nakagawa and Cuthill 2007).

Results

Physiological responses

H. wrightii showed a significant increase in aboveground biomass in clipped treatments for young patches, with an effect size of 2.81 (Figure 2). Medium patches tended to increase their aboveground biomass but no significant differences were recorded compared to control patches. Belowground biomass was not significantly different between control and

clipped treatments, but young patches tended to have the highest values (in control and clipped treatments) (Figure 3, Table 1).

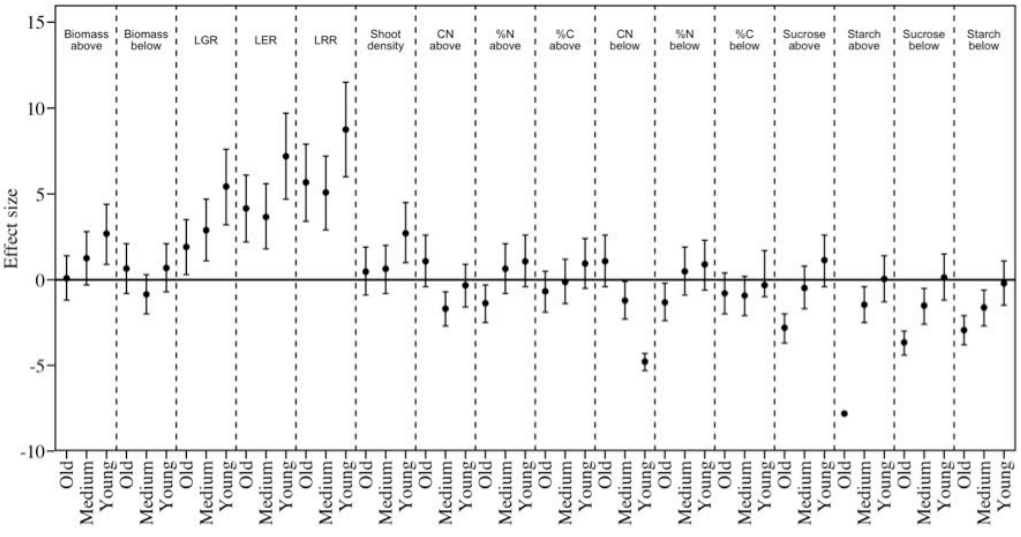

Figure 2. Effect size (n=3) for studied variables in *H. wrightii* patches in control and clipped plots in the three different colonization states: from 2010 (old), from 2012 (medium) and from 2014 (young).

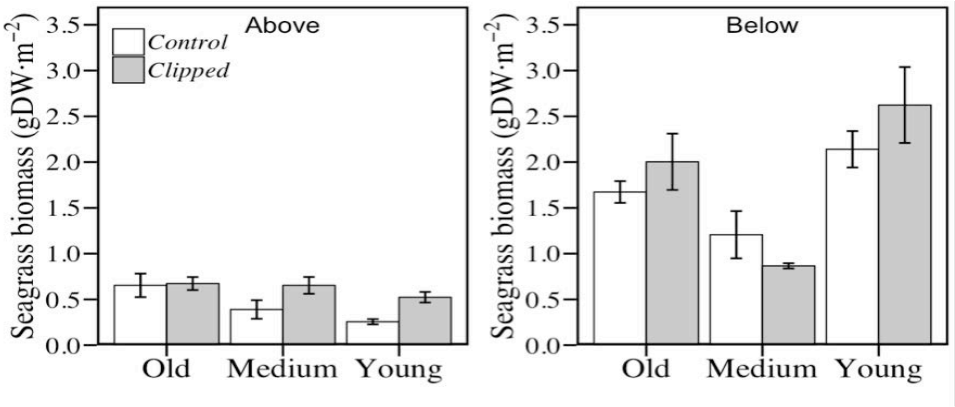

Figure 3. Above and belowground biomasses (gDW m^{-2}) of *H. wrightii* in control and clipped plots in the three different colonization states: 2010 (old), 2012 (medium) and 2014 (young). Data are represented as a mean ± s.e.

Table 1. Results of the two-way ANOVA analysis for above and belowgound biomasses. The main factors were: colonization states (old/medium/young) and treatment (control/clipped). Bold numbers indicate statistically significant results ($p < 0.05$).

Anova 2-ways test				
Above biomass	**df**	**MS**	**F**	**p-value**
Patches	2	0.11	5.03	**0.025**
Treatment	1	0.15	6.78	**0.023**
Patches:Treatmnet	2	0.03	1.34	0.29
Residuals	12	0.022		
Below biomass				
Patches	2	2.74	14.18	**0.00069**
Treatment	1	0.11	0.57	0.46
Patches:Treatmnet	2	0.28	1.48	0.26
Residuals	12	0.19		

Leaf growth rate (LGR) increased significantly in the three different patches that experienced the induced herbivory (Figure 4, Table 2), with an effect size of 1.91, 2.88 and 5.43 for old, medium and young patches respectively (Figure 1). The young patches experimented the highest growth response, increasing 5.6% than the control treatment (Table 3). Meanwhile, medium patches showed the lower differences in growth rates for the two treatments (control *vs* clipped) (Figure 4A, Table 2). The same responses were found for both LER and LRR, where young patches had the greatest responses under simulated herbivory (Figures 4B and C, Table 2). However, in control treatments no significant differences for any physiological variable among patches with different colonization states were recorded (Table 2). Otherwise, shoots density showed significant differences between the three patches in control treatments. Old patches bear the highest shoot density, and although all patches responded positively when clipped, only young patches showed a significant increase in shoot density in clipped treatments, with an effect size of 2.70 (Figure 4D, Table 2).

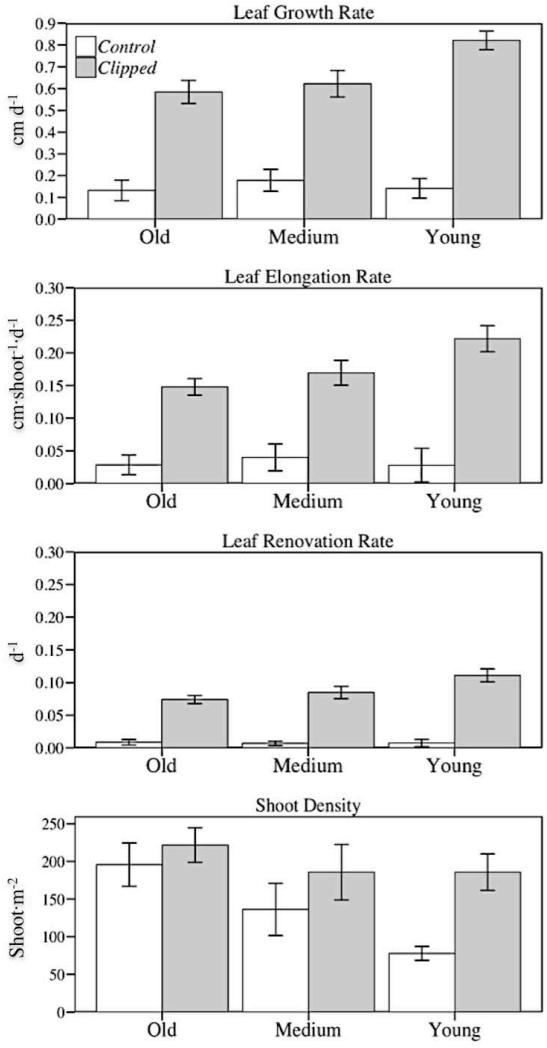

Figure 4. A) Leaf Growth Rate (cm $\cdot d^{-1}$), B) Leaf Elongation Rate (cm $\cdot shoot^{-1} d^{-1}$), C) Leaf Renovation Rate (d^{-1}) and D) Shoots Density (shoot $\cdot m^{-2}$) of *H. wrightii* in control and clipped plots in the three different colonization states: from 2010 (old), from 2012 (medium) and from 2014 (young). Data are represented as a mean ± s.e.

Table 2. Kruskall Wallis test analysis for Leaf Growth, Elongation and Renovation rates of *H. wrightii*, and Anova 2-ways test analysis for Shoots Density. The main factors were: Colonization states (old/medium/young) and treatment (control/clipped). Bold numbers indicate statistically significant results ($p < 0.05$).

Kruskall-Wallis test				
Leaf Growth	df	X^2	p-value	
Patches	2	3.422	0.18	
Treatment	1	62.99	**<0.001**	
Patches:Treatmnet	5	69.19	**<0.001**	
Leaf Elongation Rate	df	X^2	p-value	
Patches	2	2.12	0.34	
Treatment	1	56.17	**<0.001**	
Patches:Treatmnet	5	61.17	**<0.001**	
Leaf Renovation Rate	df	X^2	p-value	
Patches	2	1.48	0.47	
Treatment	1	64.94	**<0.001**	
Patches:Treatmnet	5	69.13	**<0.001**	
Anova 2-ways test				
Shoot Density	**df**	**MS**	**F**	**p-value**
Patches	2	9026	3.95	**0.048**
Treatment	1	16744	7.33	**0.019**
Patches:Treatmnet	2	2654	1.61	0.34
Residuals	12	2286		

Table 3. Average and s.e. of leaf growth for both three different patches and the % growth between treatments.

Patches	Treatment	Leaf Growth Average	se	%
Old	Control	0.133	0.047	4.4
Old	Clipped	0.585	0.053	
Medium	Control	0.179	0.051	3.5
Medium	Clipped	0.622	0.061	
Young	Control	0.142	0.045	5.8
Young	Clipped	0.821	0.042	

Biochemical responses

In terms of biochemical traits, the clipped treatments caused an overall reduction (relative to the control) in nitrogen content (Figure 5), significantly affecting leaves (effect size of -1.38), rhizomes and roots (effect size of -1.33) in the old patches (Figure 1). In contrast, nitrogen content tended to be higher in the leaves of plants from young patches relative to control treatment. Carbon content in leaves and rhizomes/roots tended to be lower in the clipped treatment for old and medium patches unlike the young patches, although significant differences were not recorded. Overall, these changes resulted in increased C:N ratios for old patches while medium and young patches remained stable (Figure 5, Table 4).

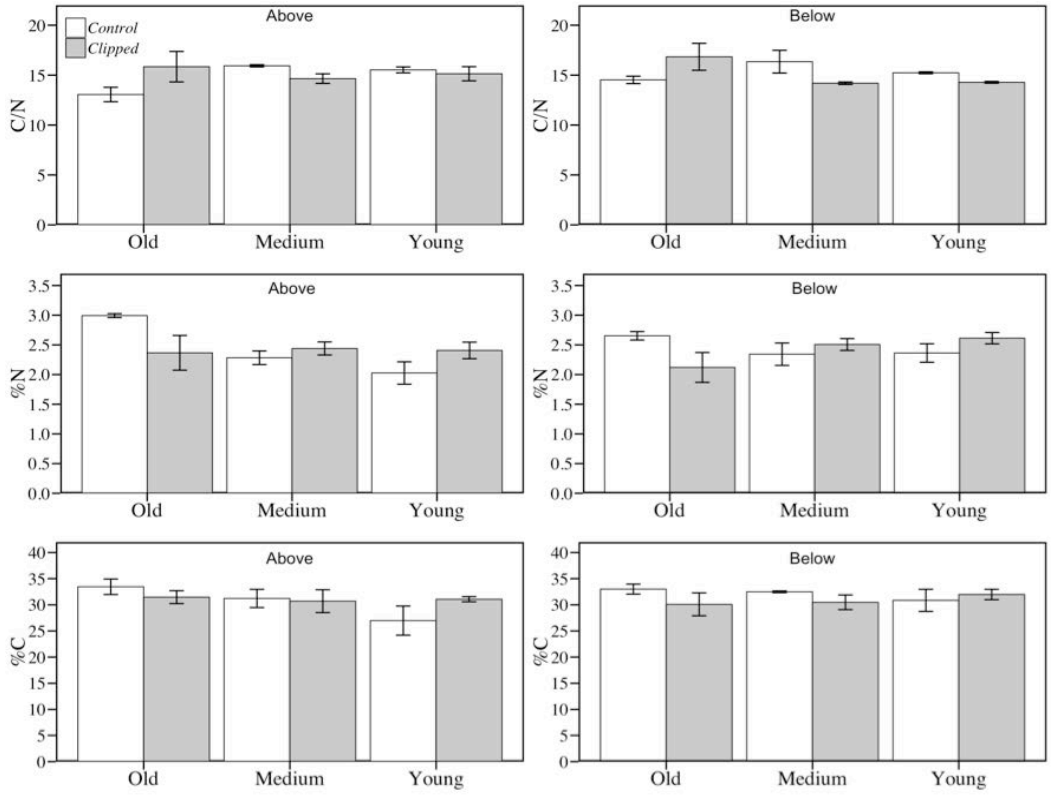

Figure 5. C:N, %N and %C of *H. wrightii* in control and clipped plots in the three different colonization states: from 2010 (old), from 2012 (medium) and from 2014 (young).

Table 4. Kruskall Wallis test analysis for C:N ratio in belowground biomass of *H. wrightii*, and Anova 2-ways test analysis for the rest of variables. The main factors were: Colonization states (old/medium/young) and treatment (control/clipped). Bold numbers indicate statistically significant results ($p < 0.05$).

Kruskall-Wallis test			
C:N Below Biomass	**df**	**X^2**	**p-value**
Patches	1	0.703	0.401
Treatment	2	0.421	0.81
Patches:Treatment	5	5.91	0.314

Anova 2-ways test				
C:N Above Biomass	**df**	**MS**	**F**	**p-value**
Patches	2	1.479	0.805	0.47
Treatment	1	0.623	0.339	0.571
Patches:Treatment	2	6.86	3.732	0.054
Residuals	12	1.838		
%N Above Biomass	**df**	**MS**	**F**	**p-value**
Patches	2	0.337	4.051	**0.045**
Treatment	1	0.004	0.049	0.829
Patches:Treatment	2	0.419	5.039	**0.026**
Residuals	12	0.0832		
%N Below Biomass	**df**	**MS**	**F**	**p-value**
Patches	2	0.015	0.215	0.809
Treatment	1	0.007	0.09	0.759
Patches:Treatment	2	0.276	3.761	0.053
Residuals	12	0.073		
%C Above Biomass	**df**	**MS**	**F**	**p-value**
Patches	2	17.725	1.815	0.726
Treatment	1	1.253	10.128	0.205
Patches:Treatment	2	15.082	1.544	0.253
Residuals	12	9.766		
%C Below Biomass	**df**	**MS**	**F**	**p-value**
Patches	2	0.026	0.004	0.996
Treatment	1	7.195	1.095	0.31
Patches:Treatment	2	6.705	1.021	0.39
Residuals	12	6.568		

The TNC content decreased significantly more than 2-fold in above and belowground parts in old patches, while in medium patches tended to be lower but not significantly different (Figure 6, Table 5), showing an effect size of -1.51 and -1.63 for sucrose and starch in belowground biomass respectively (Figure 1). In young patches, no significant differences in sucrose and starch content were recorded (Figure 6, Table 5).

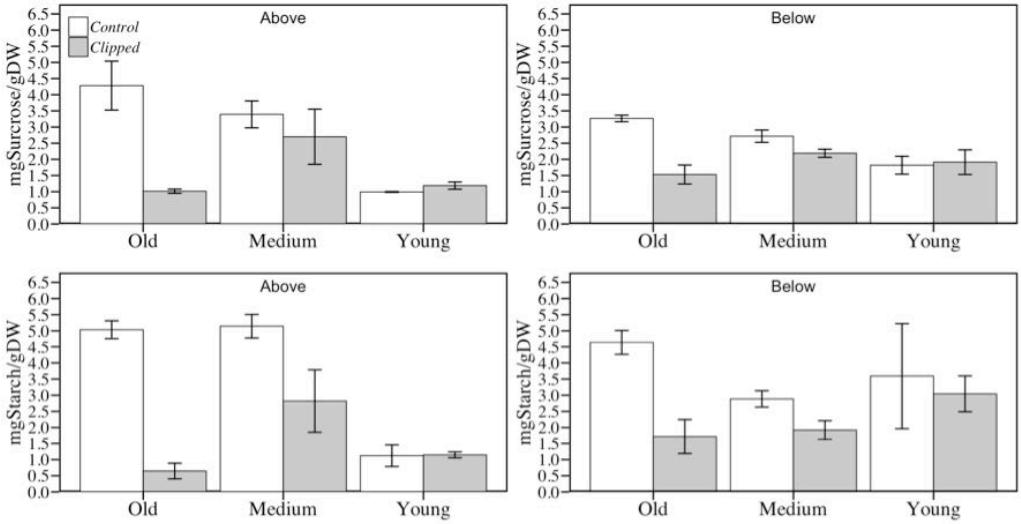

Figure 6. Sucrose and Starch (mg/gDW) for above and belowground *H. wrightii* biomass in control and clipped plots in the three different colonization states: from 2010 (old), from 2012 (medium) and from 2014 (young).

Table 5. Kruskall Wallis test analysis for sucrose (mg·gDW^{-1}) aboveground biomass of *H. wrightii*, and Anova 2-ways test analysis for the rest of variables. The main factors were: Colonization states (old/medium/young) and treatment (control/clipped). Bold numbers indicate statistically significant results ($p< 0.05$).

Kruskall-Wallis test				
Surcrose Above Biomass	**df**	**X^2**		**p-value**
Patches	1	0.86		0.353
Treatment	5	69.19		0.0647
Patches:Treatment	5	12.55		**0.027**
Anova 2-ways test				
Starch Above Biomass [(a)]	**df**	**MS**	**F**	**p-value**
Patches	2	1.2	16.42	**0.0001**
Treatment	1	2.16	29.55	**0.0003**
Patches:Treatment	2	0.86	11.76	**0.0014**
Residuals	12	0.073		
Surcose Below Biomass	**df**	**MS**	**F**	**p-value**
Patches	2	0.63	3.435	0.066
Treatment	1	2.35	12.76	**0.003**
Patches:Treatment	2	1.29	7.041	**0.009**
Residuals	12	0.18		
Starch Below Biomass	**df**	**MS**	**F**	**p-value**
Patches	2	1.45	0.82	0.46
Treatment	1	9.84	5.57	**0.036**
Patches:Treatment	2	2.4	1.36	0.29
Residuals	12			

(a) Data Sqrt(x) transformed to satisfy parametric test assumptions

Discussion

Our results showed differential compensatory responses in patches with different ages of *Halodule wrightii* when subjected to simulate herbivory. The same approach (i.e. simulating herbivory in stands with natural levels of grazing) has been used previously and results were considered to be representative of the potential response of the species when grazed (Cebrián and Duarte 1998; Moran and Bjorndal 2005; Kuiper-Linley et al. 2007; Vergés et al. 2008; Christianen et al. 2012; Sanmartí et al. 2014). Clipped treatments showed compensatory responses as denoted by the increase of biomass, shoots density, leaf renovation and growth rates (Figures 2 and 3). Our results are in agreement with those studies reporting compensatory mechanisms in some tropical seagrasses like *Thalassia testudinum* (Valentine et al. 1997; Moran and Bjorndal 2005), in the temperate species *Posidonia oceanica*

(Vergés et al. 2008; Sanmartí et al. 2014) and in *P. sinuosa* (Burnell et al. 2013). Despite herbivore damage, plant responses are based on less (undercompensation), equal, or even greater growth (overcompensation) than unattacked plants (McNaughton 1983; Belsky 1986; Oba et al. 2000; Li et al. 2010). Overcompensation is a mechanism considered more common in fast-growing than in slow-growing species (Coley et al. 1985; Haukioja and Koricheva 2000), such as the pioneer *H. wrightii* species (Zieman et al. 1989; Gallegos et al. 1994; Rasheed 2004). However, we presented the first observation about the differential compensatory response not only depending on the fast- or slow-growth, but also depending on the age of the *H. wrightii* patches. Patches from 2014 (young patches) presented the highest compensatory response in leaf growth rate (5.6 fold respect to 4.4 and 3.5 fold for patches from 2010 and 2012 respectively; Table 3). In addition, young patches showed the largest responses in shoots density and leaf renovation rate (Figure 4, Table 2). These results are consisted with (Peralta et al. 2005) who found an increase of shoots density and productivity in *Zostera noltei* populations after a severe physical impact.

Although all patches responded to simulate herbivory by increasing shoots density and stimulating leaf growth, only young patches increased nitrogen content in leaves significantly. Vergés et al., (2008) showed that the mobilisation of nitrogen within the plant facilitated the compensatory growth in *Posidonia oceanica* plants. Hence, our findings also demonstrate that enhanced growth induced by simulated herbivory also resulted in a substantial translocation of this nutrient from rhizomes and roots in young patches (Figure 5, Table 4). On the other hand, the decrease in N content not only in leaves, but also in rhizomes and roots in old patches, suggests a mobilization of nutrients to maintain leaf growth rates and to support the production of new modules (leaves and shoots) (Valentine et al. 1997). Consequently, this depletion of N may have consequences for the palatability of the tissues, as the intense defoliation caused a decline in the nutritional quality of leaves and rhizomes by increasing C:N ratios (Bjorndal 1980; McGlathery 1995; Cebrián and Duarte 1998; Goecker et al. 2005) acting as a plant defense against grazing (Augner 1995). Nevertheless, the compensatory responses reported in old patches are not only facilitated by N mobilization, but also by the use of non-structural carbohydrate reserves (TNC). Because *H. wrightii* has relatively little rhizome storage capacity, it may be particularly sensitive to external alterations (Onuf 1996; Hauxwell et al. 2001). Indeed, mobilization of carbohydrate reserves appears to play a major role in the ability of plants to withstand disturbances involving the loss of aboveground tissue (Rodgers et al. 1995; Brun et al. 2003; Eklöf et al. 2008). However, the magnitude of the contribution of carbohydrates to regrowth may depend on both storage capacity and physiological integration of the plant. The depletion of TNC after clipping in old patches suggests that carbohydrate mobilization took place in response to defoliation, and part of the compensatory leaf growth (Figure 6, Table 5).

In summary, *H. wrightii* can exhibit differential plasticity in response to herbivore stress depending on colonization states. Old patches can induce substantial compensatory responses, such as enhanced growth through mobilisation of internal reserves. In contrast, in young patches there is no trade-off between compensatory growth and a cost for internal reserves. This is the first study to simultaneously examine compensation traits on seagrasses after a natural recovery, and results show that young patches are less vulnerably to grazing attack. Finally, our findigings suggest that herbivores could have an important role in seagrass community structure and function, and could be a key force during succession dynamics.

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GENERAL DISCUSSION

Acepta, deja fluir. No hay nada absoluto, todo cambia, todo se mueve, todo revoluciona, todo vuela y se va…
Accept, let everything flow. There is nothing at all, everything changes, everything moves, everything turns upside down, everything flies and advances…

Frida Kahlo

Influence of hydrodynamics on seagrass-grazers interaction

Hydrodynamics effects on feeding and foraging behaviour of seagrass consumers

This Thesis has demonstrated how hydrodynamic conditions play an important role in seagrass-grazers interactions, limiting the feeding capacity of mesograzers (**chapter 1**) as well as the direct consumption of seagrass leaves (**chapter 2**). Firstly, we hypothesized that hydrodynamic conditions may affect the feeding and foraging behaviour of mesograzers in seagrass communities. This hypothesis was demonstrated by the observations of the feeding behaviour of *Gammarus locusta* under controlled conditions, where the higher the velocity the lower the spanning time feeding and the lower the swimming capacity, which resulted in lower global rates of epiphyte removal. In addition, *in situ* measurements using the direct tethering method at four locations characterized by different hydrodynamic regimes reinforced these findings, since the highest rates of annual defoliation occurred at the most sheltered site. In contrast, lower defoliation rates were found in the most exposed site. Interestingly, these patterns were also found for the detrital dynamics, although this data are not presented in this Thesis (Table 1).

"The feeding and foraging behaviours of seagrass mesograzers are affected by the combination of hydrodynamics and contrasting habitat complexity (shoot density). This is significant because the outcome of such interactions depends on the strength and magnitude of both biotic and abiotic variables." ***Chapter 1.***

Table 1. Summary of the variables studied in chapters 1 and 2, and values of a yet unpublished work examining detrital dynamics of *Cymodocea nodosa* under different hydrodynamic regimes in laboratory and *in situ* conditions. Data are expressed as a mean ± s.e.

CHAPTER 1			
Flow velocity	Feeding rate in high density artificial beds ($\mu gChla \cdot mgDW^{-1} \cdot h^{-1}$)	Feeding rate in low density artificial beds ($\mu gChla \cdot mgDW^{-1} \cdot h^{-1}$)	
Low	0.035 ± 0.015	0.018 ± 0.014	
Medium	0.024 ± 0.012	0.0042 ± 0.003	
High	0	0	
CHAPTER 2			
Experimental sites from low to high hydrodynamics exposure	Mean production ($cm^2 \cdot shoot^{-1} \cdot d^{-1}$)	Mean defoliation ($cm^2 \cdot shoot^{-1} \cdot d^{-1}$)	% Defoliation
CC	0.818 ± 0.034	0.606 ± 0.33	74.08
ST	0.893 ± 0.042	0.603 ± 0.34	67.52
BC	0.676 ± 0.18	0.389 ± 0.256	57.54
CH	0.616 ± 0.12	0.287 ± 0.261	46.59
UNPUBLISHED WORK			
Experimental sites from low to high hydrodynamics exposure	k (d^{-1})	$T_{1/2}$	Turnover
CC	0.041 ± 0.015	36.93 ± 72.97	53.29 ± 105.27
ST	0.032 ± 0.016	86.72 ± 71.16	125.12 ± 102.66
BC	0.021 ± 0.016	89.32 ± 51.43	129.96 ± 74.19
CH	0.012 ± 0.008	92.83 ± 52.27	133.92 ± 75.42

The decay constant, k(d^{-1}), is calculated by $W_t = W_o \cdot e^{-kt}$, where Wt is the weight of material left from initial weight, W0 after time t. The litter half-life is calculated as $T_{1/2} = \ln 2 \cdot k^{-1}$ and Turnover = 1/k

Decomposition rates (k, d^{-1}), half time ($T_{1/2}$) and turnover in the same studied locations used in chapter 2 for the detrital biomass of *Cymodocea nodosa* are given in Table 1. As for the decomposition rates of living tissues, a negative relationship was observed between flow velocity and decomposition rates (Jiménez-Ramos et al., in preparation a). Probably, this negative relationship is due to the physical stress over consumers promoted by hydrodynamic conditions, since lower hydrodynamics may allow organisms to invest a higher fraction of their energy and time in swimming and traveling among shoots to feed over them. Thus, in patches where conditions are more beneficial, organisms can move more frequently, resulting in aggregation in these areas and even to have the opportunity to selectively feed on the leaves. In fact, the amphipod *G. locusta* tended to aggregate at the downstream leading edge of the reconstructed bed, where flow velocity is known to be highly reduced by seagrass canopy (Morris et al. 2008, González-Ortiz, et al. 2014). Moreover, at low velocity treatments, *G. locusta* individuals positioned in dispersal positions along the flume, matching with a higher percentage of, individuals swimming (from a 23% -33% of individuals) within the canopy. Hence, these findings clearly indicated that seagrasses acted as foundation species, having a positive biological effect on mesograzers through a facilitation process (i.e. reducing hydrodynamic pressure), which did not only have a direct impact on mesograzers, but also in the foundation species (i.e. reducing epiphyte load).

Habitat complexity constraining hydrodynamic influences on feeding rates

On the other hand, this Thesis demonstrated the existence of an interrelation between hydrodynamics and habitat complexity and the feeding and foraging behaviour of seagrass grazers. **Chapter 1** showed how the relationship between hydrodynamics and shoot density influenced the feeding and foraging behaivour of mesograzers, observing higher feeding rates and vigorous swimming behaviour at higher density. In contrast, when high flow velocity conditions were combined with low habitat complexity, *G. locusta* consumption was strongly reduced. These evidences were reinforced in the findings of **chapter 2**, where denser meadows inhabiting sheltered areas supported the highest defoliation rates. Moreover, in unsheltered sites, the highest rates of defoliation were recorded when shoot density of *C. nodosa* meadows was high.

"Our work demonstrates that the trigger of trophic cascade effects depends on the strength of the interrelationships and feedbacks among seagrasses (e.g. habitat complexity), abiotic (e.g. flow velocity) and biotic (e.g. epiphytes and mesograzers) compartments". ***Chapters 1 - 2.***

Several studies have observed the consequences of seagrass meadows as flow modulators on top-down control, since they can change the physical environment through their physical structures (Bouma et al. 2005, Koch et al. 2006). This role as ecosystem engineers can results in positive (e.g. facilitation) or negative effects on the survival of associated species (Bertness and Callaway, 1994; González-Ortiz et al., 2014a) and can affect the occurrence of filter- feeding infauna (e.g. distribution, survival, growth, etc.) (Dang et al., 2010; González-Ortiz et al., 2016; González-Ortiz et al., 2014b; Irlandi, 1996). However, these studies worked with benthic species and not with mobile consumers in the water column, which can feed directly from leaves and epiphytes. Novelty, this Thesis reports on mobile consumers within the canopy, and these findings reinforce the importance of seagrasses as foundation species, having a positive biological effect on mesograzers through facilitation (i.e. reducing hydrodynamic pressure).

Leaf traits influence on seagrass-grazer interactions

Hydrodynamics modulate seagrass palatability by intra-specific differences in leaf traits

Through this Thesis the existence of intra-specific differences relative to seagrass acclimation to different hydrodynamic conditions leading to modifications in leaf palatability was demonstrated (**chapters 2** and **3**). Our hypotheses focused on the acclimation of seagrasses to the marine environment or human disturbance may provide intraspecific differences relative to leaf properties and thus, influencing on the leaf palatability.

Our results showed intra-specific differences in several leaf traits from *Cymodocea nodosa* and *Zostera noltei* inhabiting different locations of Cádiz bay, which is in agreement with previous results from De los Santos et al. (2013), who also demonstrated the existence of intraspecific differences in leaf traits on the same temperate species in the same locations. Consequently, seagrass leaves differed in their value as food (i.e. palatability), varying in morphology, nutritional and structural leaf traits. Overall, plants from sheltered sites had higher nutritional quality (e.g. N content) and lower structural and biomechanical defences

(e.g. carbon and fiber contents), being therefore more palatable to herbivores. It is not surprising therefore, that seagrass consumer's had the capacity to feed selectively those leaves more palatable within the pool of leaves with intra-specific differences on leaf traits. Interestingly, in **chapter 2** there was a selective behaviour of mesograzers towards the most palatable leaves in the most sheltered place (that is, CC), reinforcing the influence of hydrodynamics on the feeding behaviour of consumers. In contrast, in **chapter 3** we examined the influence of inter and intra-specific differences on leaf traits of two temperate species *C. nodosa* and *Z. noltei* on leaf selection by the generalist herbivore *Paracentrotus lividus*. The two seagrass species varied among the different morphometric, nutritional and structural traits, both inter- and intra-specifically, according to the different conditions from inner (sheltered site) and outer bay (exposed site). Sea urchins consumed significantly less *C. nodosa* from the inner bay, which had greater structural defences, reinforcing the increasingly recognized role of structural defences against seagrass grazing (Pennings et al. 1998, Marinai and Alcoverro, 1999, Vergés et al. 2007a, Prado and Heck 2011).

On the other hand, these findings highlighted the importance of studying different types of consumers, since each herbivore species has a feeding behaviour, a way of eating and a different mobile capacity, which should modify the consumption of one or another type of tissue under different influencing factors (biotic or abiotic). In fact, Bakker et al. (2016) grouped herbivores according to diet, habitat selection and movement ecology, and demonstrated a differential functional role of each studied herbivore on submerged plant abundance and species composition. This approach could have consequences in the face of the loss of biodiversity that we meet today (Cardinale et al. 2012). In this vein, Blake and Duffy (2010) showed a positive impact of multi-species invertebrate grazer assemblages in eelgrass systems, which had the potential to buffer some important ecosystem properties against increasingly common stressor impacts (e.g. warming, nutrient or salinity inputs). Such response to grazer diversity may become increasingly important as managers seek to maintain ecosystem properties in the face of climate and other environmental change impacts.

What is the main leaf trait affecting herbivore preference over seagrass leaves?

Previous studies on plant-herbivore interactions and food-preferences of herbivores in seagrass ecosystems have commonly reported that nutritional, structural and chemical traits may determine leaf palatability and regulate plant quality as food (Zapata and McMillan 1979, Mariani and Alcoverro 1999, Cebrian et al. 2009, Prado and Heck 2011). Nutritional quality is considered the main factor regulating feeding decisions by consumers in terrestrial and aquatic ecosystems (Elser et al. 2000, Cebrian et al. 2009). However, experimental evidences have

shown that the response of herbivores to leaf nitrogen content is not straightforward on seagrass communities (Bjorndal 1980; Zieman et al. 1984; Klumpp et al., 1989; McGlathery 1995; Marini and Alcoverro, 1999; Goecker et al. 2005). Recent publications on traits mediating feeding choices of seagrass consumers suggest that structural traits of seagrass leaves (related to leaf mechanical resistance) may play an important role in regulating the plant-herbivore interactions in seagrass ecosystems (e.g. Prado and Heck 2011; Vergés et al. 2011).

"The intra-specific differences on Cymodocea nodosa (from inner vs outer bay) reinforce the increasingly recognized role of structural defenses against herbivory" ***Chapter 3***

Throughout this Thesis the importance of structural defenses in the feeding decision done by the generalist herbivore *Paracentrotus lividus* was highlighted. Although nutritional quality is an important indicator of the susceptibility to be consumed (Goecker et al. 2005, Cebrián et al. 2009) as it has been recorded in *C. nodosa* plants subjected to high nutrient concentrations (**chapters 4** and **5**), the structural defences seemed to be the main trait determining the feeding choice done by *P. lividus* in **chapters 3** and **4**. Otherwise and intimately linked to structural traits is the influence of the biomechanical properties on seagrass palatability (**chapters 2**, **4** and **5**). Although few studies worked directly on the relationship between biomechanical traits and feeding preference by seagrass consumers (e.g. Martínez-Crego et al., 2016), some studies have hypothesized on the possible effect that the biomechanical traits of leaves could have on feeding preferences by herbivores (De Los Santos et al. 2012). Our results have shown similar patterns in **chapters 2**, **4** and **5**, where weaker leaves are more palatable to herbivores and therefore, more consumed. These results suggest that a reduction of mechanical resistance could be an important force contributing to the decline of seagrass meadows and associated species (Hughes et al. 2009) as it has been pointed out in previous studies (La Nafie et al. 2012).

On the other hand, chemical defenses have been commonly pointed out as determinants of food choice by most herbivores in marine systems (Bolser and Hay 1996, Prusak et al. 2005, Vergés et al. 2007b). For example, Vergés et al. (2007b) demonstrated that chemical defenses from the seagrass *Posidonia oceanica* dramatically reduced the feeding of a wide range of consumers, including fishes and sea urchins. More recently, Tomas et al., (2011) found that herbivorous isopods preferred tissues of the eelgrass *Zostera marina* bearing low concentration of phenolic compounds. Although in this Thesis there is not an in-depth study

of the role of chemicals in the food preferences of seagrass consumers, phenolic compounds not previously reported for *C. nodosa* have been identified (see **chapter 5**).

"A notable accomplishment of this work is the identification of phenolic compounds not previously reported for Cymodocea nodosa" ***Chapter 5***

Under these considerations, the results of this Thesis indicate that herbivore preference over seagrass tissues cannot be only described by a main leaf trait, but by the combination of all those factors contributing to tissue palatability, as well as the feeding and foraging behaviour of consumers (Boström and Mattila, 1999; Prado and Heck, 2011; this thesis) or the abundance and distribution of other macrophytes dwelling seagrass ecosystems.

Consequences of macrophyte diversity in seagrass communities

Seagrasses co-occur with other primary producers (e.g. seaweeds, epiphytes, etc.) creating mixed communities, and therefore herbivores have access to several food sources in a seagrass based ecosystem (Hulme 1996). Consequently, if herbivores have the capacity to make a choice among the different available food sources, it may generate different patterns of vegetation consumption within the ecosystem, which may increase or decrease the direct consumption of seagrass tissues. Hence, we hypothesized that within a seagrass community, a preferential consumption of some species *versus* others may exist depending on the palatability of their tissues, and the susceptibility of seagrass species to be grazed can also show a temporal and spatial variability, as we demonstrated in **chapter 2**. To test this hypothesis, we explored the existence of a preferential consumption of some species *versus* others in a seagrass community, depending not only on the palatability of their tissues, but also on the abundance of other edible species (**chapter 3**). The most consumed species was *Ulva* sp., which presented the highest N content and OM (high nutritional quality) joined with low structural reinforcement (i.e. low C and fiber contents), making it one of the most palatable species. Based on our findings, we verify the belief that most macroalgae are usually more consumed than seagrasses, since in the latter their high fiber content and lower nutritional quality make them less palatable (Valiela 1995). This pattern was similar in the acquired results from **chapters 4** and **5**. However, in these chapters we also demonstrated an increase of nutritional quality in *C. nodosa* leaves, derived of nutrient acclimated period, joined to an increase of seagrass consumption. The seagrass consumption also increased by the epiphyte presence, which may further strengthen the negative effects promoted by eutrophication phenomenon. This may has great ecological implications, since during eutrophication events

plants are subjected to indirect (e.g. light reduction promoted by ephemeral algae growth, anoxic conditions, etc) and direct effects (e.g. ammonium toxicity) affecting negatively their growth and survival, (Brun et al. 2002, 2003, Burkholder et al. 2007). Therefore, these findings indicated the importance of the proliferation of other ephemeral macroalgae, such as the genus *Ulva* during eutrophication events, which tend to be more palatable to consumers, reducing the direct consumption over seagrasses. Thus, the presence of ephemeral macroalgae may improve the seagrass resilience under eutrophication events, or even under natural disturbance such as El Niño (Mazariegos-Villarreal et al. 2012). However, given the potential influence of herbivores on macrophyte distribution and abundance (Steneck and Sala 2005), future studies should incorporate observations of feeding behaviour for a better understanding of macrophyte-herbivore interactions.

Seagrass under threats: the effects over herbivory process

Seagrass communities are under threat from eutrophication and co-occurring global stressors (e.g., ocean acidification, rising temperatures), which can modify seagrass–grazers interaction, both directly by affecting the physiology of the grazers, and also indirectly by altering the leaf traits of the plants, and thus changing leaf palatability (Cruz-Rivera and Hay 2000, O'Connor 2009, Tomas et al. 2015). In **chapters 4** and **5** we demonstrated how aforementioned stressors lead to an increase of the palatability of seagrasess through the modification of leaf traits and enhancing epiphyte abundance.

Eutrophication and the epiphyte paradigm

One of the main threats to seagrasses come from eutrophication, or nutrients enrichment, indirectly or directly affecting them (Burkholder et al. 2007; Brun et al. 2003). In this Thesis an increase in the nutritional quality of seagrass leaves under elevated nutrient concentrations was shown, which enhanced the intensity of herbivory on these tissues (**chapters 4** and **5**). Moreover, increased eutrophication often leads to the proliferation of epiphytes in leaves of seagrasses, which can outcompete for light and nutrients (Dennison et al. 1993, Hauxwell et al. 2001) and the above-mentioned ephemeral macroalgae, such as *Ulva* sp (Burkholder et al. 2007; Chávez-Sánchez et al. 2017).

"... by the presence of ephemeral macroalgae such as Ulva sp., the herbivory pressure over seagrass could be turned improving seagrass resilience to eutrophication events". ***Chapter 4***

In this Thesis, food choice experiments with fresh leaves and agar diets revealed a strong evidence of the role of epiphytes in increasing direct consumption of *C. nodosa* leaves by *P. lividus*. Regarding Mediterranean herbivores, past studies have indicated that *P. lividus* preferentially feed on the epiphytes of *P. oceanica* leaves (Boudouresque and Verlaque 2001, Tomas et al. 2005). Thus, in **chapter 4** we showed how the presence of epiphytes in leaves can stimulate the direct consumption of the temperate seagrass *Cymodocea nodosa*. This preference may be lead because epiphytes growing on seagrass leaves have higher nutritional quality than leaves (Alcoverro et al. 2000), which may lead to an increase of the feeding selectivity by herbivores (Marco-Méndez et al. 2015). As a consequence, the reader might think that the known positive effect exerted by grazers because of the reduction in the epiphytes coverage in seagrass leaves (Hughes et al. 2004) under high nutrients load (Orth and Van Montfrans 1984), could become negative for seagrass populations, because of the increase in the direct seagrass leaf consumption, leading to a higher biomass loss due to herbivory. However, it should be noted that feeding choice assays do not simulate natural conditions totally, eliminating an important factor such as hydrodynamics. In this thesis it has been observed how the consumption of epiphytes and leaves can be reduced by hydrodynamic stress over mesograzers. On the other hand, it has been demonstrated that currents have an effect of washing out epiphytes especially in conditions of high hydrodynamics (**chapter 1**), regulating the epiphyte cover over leaves. In conclusion, our results highlight the role of epiphytes as enhancers of nutritional quality of tissues and then, in increasing direct consumption of *C. nodosa* leaves, and the need to deepen on interactions between hydrodynamics, epiphytes and grazers in seagrass ecosystems.

Global change effects under multifactorial view

In a context of global change and increasing occurrence of human-induced stresses (Easterling 2000), it is important to understand how changes in abiotic conditions can modify the outcome of species interactions while being able to scale up these changes to the community level. This challenge requires a multi-scale approach from individual responses to populations, communities and the ecosystem (Russell et al. 2012), taking into account that the overall effect of multiple stressors can be non-additive (Casas-Valdez et al. 2003, Woodward et al. 2010, Villazán et al. 2016). Then, in addition to the increase of seagrass consumption under eutrophication events, a potential increase of seagrass consumption was recorded under warming and acidification scenario **(chapter 5).** Others authors also found a higher consumption in seagrass acclimated to expected future conditions (Burnell et al. 2013, Tomas et al. 2015) even in seedlings (Hernán et al. 2016), but other studies considering a single factor did not achieve significant changes (e.g. O'Connor 2009). On the other hand, the acclimation of leaf traits to a global change scenario was highly variable among the three factors studied, acting together or independently. Hence, we can conclude from this study that leaf trait

responses can be highly variable, depending on whether driving environmental changes are considered as a single factor or in combination, and this will affect predictions of how these drivers will influence herbivory in ecosystems in the future.

Methodological limitations

A combination of tethering and food preference experiments were used to determine which leaf traits determined the feeding behaviour and consumption rates of herbivores. It should be noted that each method has some associated limitations that must be taken into account when interpreting the results. The estimates of defoliation rates obtained by the tethering method can be influenced by several factors such as time, space, predators, etc., causing a high variability. However, thanks to the use of a direct method as the tethering, it has been possible to demonstrate, through different studies, that the herbivory processes can determine the structure and distribution of macrophytes (Kirsch et al. 2002, Tomas et al. 2005, Taylor and Schiel 2010), as well as the spatially and temporally variability of these processes (Prado et al., 2007; Steele et al., 2014; this work in chapter 2). On the other hand, feeding preference experiments (laboratory conditions) allow testing the selectivity of the herbivores, isolating them from those factors acting in nature (e.g. hydrodynamics, predation, competition, etc.), which can help to elucidate how factors related to food palatability influence the herbivore selectivity. Therefore, the combination of both types of methodologies would generate more reliable results allowing a comprehensive view of the seagrass-grazer interactions.

Seagrass response to herbivory damage

The impact of herbivores on plant populations often creates indirect effects that influence the abundance, diversity and composition of the entire plant community (Augustine and McNaughton 1998, Olff and Ritchie 1998, Chase 2000). However, the link between the evolution of individual plant traits and the impact on the entire plant community remains elusive, in part due to the difficulty in merging these different scales of investigation. As a consequence, new steps in the light of plant responses to herbivorous attack were taken. In the last chapter of the thesis (**chapter 6**), a novel study in the seagrass responses to the herbivory, with differential responses according to different colonization states was done. The findings showed that young patches were less vulnerable to grazing damage. This suggests that young patches compensate better the herbivory in comparison to the more advanced state of the communitiy, and last ones may develop greater resistance strategies to herbivory.

Deepening in the plant response to the damage caused by grazing, others works were performed, but have not been included in this Thesis. The indirect consequences of grazing

have been studied through experiments on the N utpake (after sea urchin attack in mesocoms experiments), or scaling up at the community level this response by exploring the changes on carbon metabolism and DOC fluxes (by simulated herbivory in fieldwork).

A higher N uptake was found for grazed *C. nodosa* leaves (Figure 1) by sea urchins (Jiménez-Ramos et al., in preparation b). Since grazing damages photosynthetic tissues, plants must respond in rebuilding their tissues to maintain the growth of their biomass. To do this, plants increase the production of proteins as well as chlorophyll (Kraemer et al. 1997, Stapel and Hemminga 1997, Hemminga et al. 1999). Both compounds require large amounts of N so the enzymes involved in nitrogen assimilation should work for the rapid incorporation of N into tissues, as has been shown in plant-herbivore studies conducted in other ecosystems (Lehman and Scavia 1982, Cargill and Jefferies 1984, Bianchi 1988, Williams and Carpenter 1988, Sand-Jensen et al. 1994). Therefore, the increase in N uptake may be the results of the need to reconstruct tissues in grazed plants.

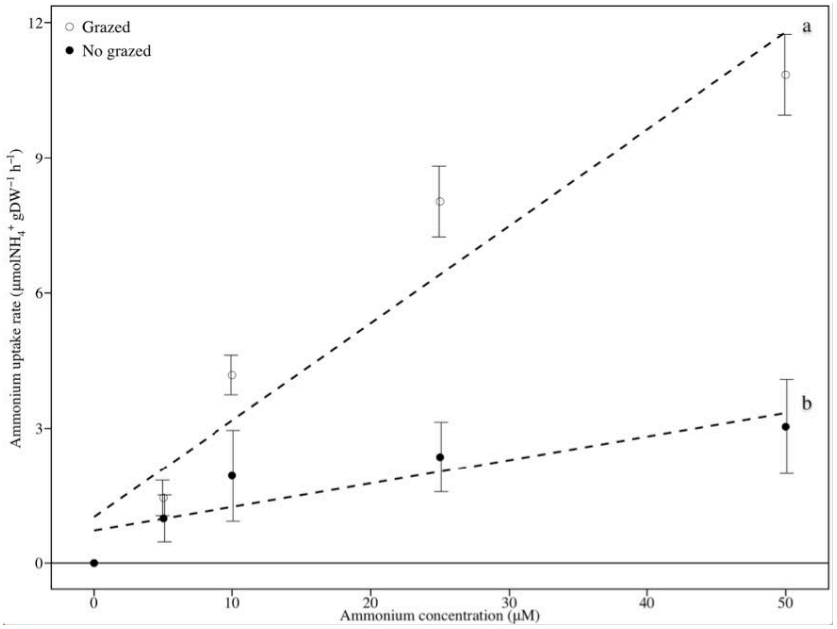


Figure 1. *Cymodocea nodosa* foliar ammonium uptake rates (μmol g^{-1} DW h^{-1}) *versus* ammonium concentration (μM) at the whole interval assayed (120 min). Letters indicate significant differences among treatments (p<0.05) from 1-way Anova test analyses. Data are presented as means ± s.e. (n = 3 replicates).

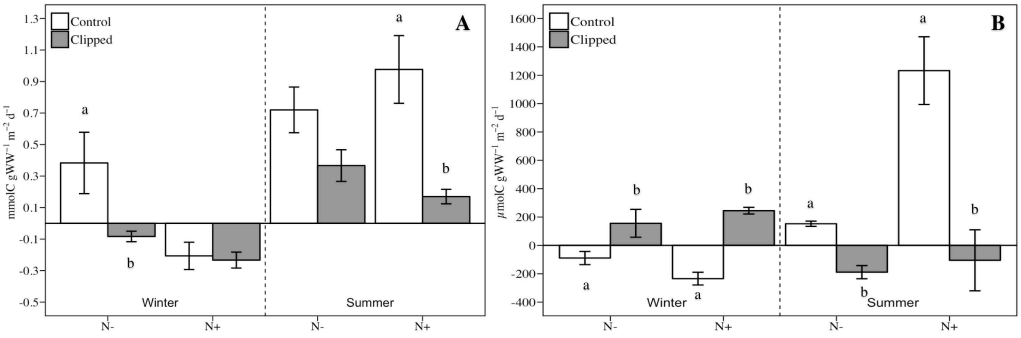


Figure 2. Effect of simulated herbivory and nutrients enrichment on A) Net Community Production and B) net DOC fluxes in winter and summer. N-: control treatment; N+: nutrients enrichment treatment. Different letters indicate significant differences between control and simulated herbivory (p<0.05) from 1-way Anova test analyses. Data are expressed as mean ± s.e. (n=3).

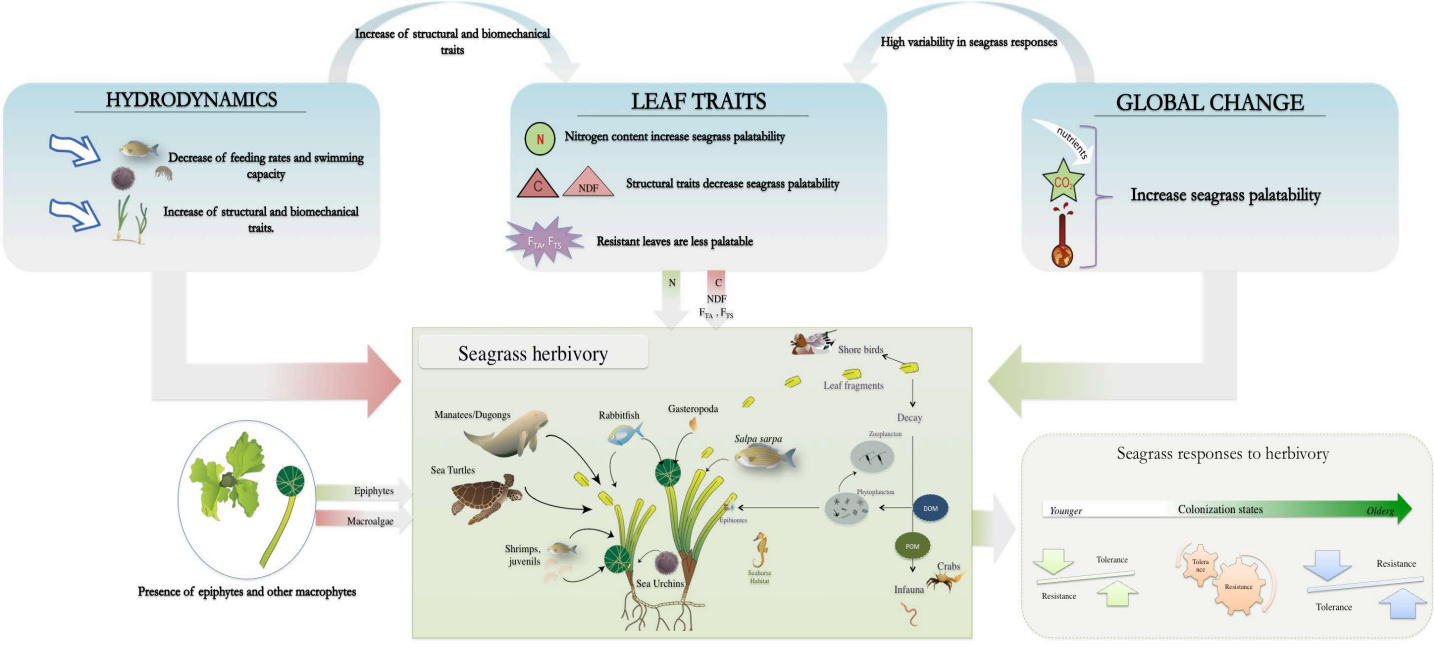
Simulated herbivory produced a decrease of the net community production (NCP) in both seasons (winter and summer), even shifting the seagrass community from autotrophic to heterotrophic in winter (Figure 2). Regarding DOC fluxes, the simulated herbivory caused opposite responses in both seasons and both control and nutrient enrichment treatments. In addition, nutrients enrichment produced a decrease in net community production (NCP) and DOC fluxes in *C. nodosa* meadows in both season, except in *C. nodosa* meadow in summer trial when a slight increase was found (x1.2 and x7 respectively) (Jiménez-Ramos et al., in preparation c). Probably, this variability is related to the changes in fauna diversity and to the increase in the abundance of fast-growing species as macroalgae. Therefore, we can conclude that herbivory pressure can potentially change both carbon metabolism and DOC fluxes in marine angiosperm communities, leading to important consequences on the transfer of matter and energy in the ecosystem.

Ecological implications

The configuration of marine communities structure is based on the concepts of top-down and bottom-up controls (Carpenter et al. 1985). These opposing but complementary concepts may be particularly useful in understanding the complex interactions between macrophytes and its herbivores. Although the bottom-up/top-down debate has been, at times, highly polarised (Slobodkin et al. 1967, White 1978), there is a general consensus that both processes are crucial to the functioning of the ecosystems (Hunter and Price 1992, Korpinen et al. 2007).

The dilemma of marine ecologists arises because different ecosystems are characterised by significant differences in the relative importance of both controls (Valiela 1995, Richards and Coley 2007). Especially, the paradigm of herbivory is to understand the relative predominance of these two controls in the functioning of marine ecosystems. Many researchers recognize that both controls can act together, determining the structure and functioning of coastal ecosystems (Lotze 2006). However, it is necessary to delve deeper into theory to a better understanding if both are responsible for the changes in the communities.

Figure 3. Schematic illustration of studied factors that influence on seagrass herbivory. Red arrows indicate a decrease of seagrass palatability and green arrows indicate an increase of seagrass palatability.



Among the several factors associated to both controls, this Thesis has focused on the study of the seagrass-grazers interaction and the factors potentially involved in seagrass consumption (Figure 3). It has been highlighted the crucial role of hydrodynamics as a regulator in the top-down control (**chapters 1** and **2**). Hydrodynamics have shown to have a strong effect on the feeding and foraging behaviour of consumers, reducing their consumption and swimming capacity at high flow, as well as their capacity to washing out epiphytes from leaves. The role of leaf traits determining their probability to be consumed has been proven in the herbivore feeding preference assays, and results underlined the relevance of nutritional and structural traits. In addition, epiphyte presence improves leaf palatability and then increased the direct consumption of leaves. Under these considerations, in sheltered areas where hydrodynamics is very low, leaves may be more palatable (low structural and biochemical defenses), herbivores do not suffer physical stress and the epiphytic washing out is minimum, the top-down control (i.e. herbivory) may have a greater relevance by regulating the community structure. In contrast, in unsheltered areas (high exposure to currents), where leaves may bear higher structural and biomechanical defenses, grazers are subjected to higher physical stress (reducing their consumption and food selectivity capacity) and also the washing out of epiphytes may occur, the bottom-up control may be more relevant regulating the structure of the seagrass community. Hence, hydrodynamics characterization in seagrass ecosystems should be a crucial point in the development of conservation and management plans, because may cause larger cascading impacts, related to the alteration of the food web, which may also be drivers of seagrass decline.

Challenges in conservation and management on seagrass ecosystems

Loss, change or species disappearance within seagrass meadows are usually correlated with decreases in light availability, eutrophication, increases in sedimentation, or direct physical disturbance (Waycott et al. 2009). Other stressors such as the presence of invasive species and disease can also lead to habitat degradation (Williams 2007; Tomas et al. 2011b). Although most seagrass losses have been driven by poor coastal zone management leading to increase in nutrients availability and decrease in water transparency (Short and Wyllie-Echeverria 1996), the potential for disruption of top-down processes to drive some of these declines has been largely overlooked (Unsworth et al. 2015).

On the other hand, the top-down process has been also overlooked on marine reserves plans, which they have emerged as an important tool in the management and conservation of marine communities. Large marine protected areas (MPAs) may be ideal for biodiversity conservation because they encompass more species. As a consequence, populations of seagrass consumers such as fish or sea urchins tend to concentrate in MPAs, resulting in enhanced grazing pressure (Vergés et al. 2008). Therefore, the inclusion of critical

seagrass areas within the design of future marine reserves should deserve more attention to prevent possible overgrazing events. This raises some key questions that require further research, such as examining the threshold of herbivory that can be tolerated by a seagrass meadow.

In light of the last point of the Thesis where the variability in herbivory damage response is studied, a more holistic view must be taken into account when formulating management and conservation plans in seagrass ecosystems (Prado et al. 2012). This Thesis showed that the response to herbivory damage is different according to the colonization states, generating new perspectives in management and conservation plans in seagrass ecosystems (**chapter 6**). The findings underscore the importance of decisions on how conservation plans are developed and generate new points to be taken into account in management plans. From our results, the importance of managing seagrass ecosystems in eutrophic areas is highlighted. In addition to the fact that the leaves are usually more consumed under eutrophication events (**chapters 2**, **4** and **5**), other potential effects such as ammonium toxicity could be developed in the presence of herbivores (Jiménez-Ramos et al., in preparation b) and affecting seagrass resilience. Moreover, seagrass communities may even become heterotrophic under overgrazing events (Jiménez-Ramos et al., in preparation c), diminishing their potential to capture carbon. Meanwhile, the increase of leaf palatability under elevated temperature and acidification and the variable leaf traits responses could affect adversely the health of seagrass populations. On the other hand, natural disturbance may also take into account because can modify the diet of seagrass consumers (Mazariegos-Villarreal et al. 2012). Hence, all of these stressors work together to reduce the resistance and resilience of seagrass meadows in the face of other global-scale environmental. Further information on how these stressors interact at the ecosystem and landscape scale to influence ecosystem resilience is needed to better understand the key pressure points so that management can be appropriately targeted.

FURTHER RESEARCH

Si la vida es como una aventura, no hay mayor fortuna que vivirla bien, por eso mi reino no es lo que tengo sino lo que hago…
If life is like an adventure, there is no greater fortune than living it well, that is why my kingdom is not what I have but what I do…

Juan Carlos Aragón (Las noches de bohemia)

In these years of intense reading, hypotheses elaboration and interpretation of results of this Thesis, a set of new hypotheses and questions have arisen. Some of them to delve into the chapters of the Thesis and others corresponding to other topics derived from the literature. In addition, through direct collaboration with other colleagues, new questions have also emerged wrapping relationships between herbivory processes with others ecological process, from a physiological perspective to a community-level or even globally view in seagrass ecosystems.

To deepen in role of hydrodynamics and habitat complexity on the feeding and foraging behaviour of the seagrass consumers

How do mesograzers withstand and utilize moving water?

Processes operating at the level of individual organisms can determine the properties of populations, communities, and ecosystems (Koehl 1984, Denny et al. 1985). Understanding how mesograzer species are biomechanically prepared to face the physical stress of the currents could improve the knowledge of its swimming behaviour and thus determine the degree to which its mobility is restricted in a hydrodynamic environment.

What factors determine the grazer's dilemma between feeding or sheltering in seagrass habitats?

The mechanisms affecting habitat choice and grazing in seagrass meadows could be summarized in the steering both food source and protection from predation (Puttman 1986). In low-risk environments, herbivores spend more time feeding in vegetation with high food quality (Puttman 1986), but in the presence of a predator, or in harsh environments, prey organisms may change behaviour or shift habitat at the cost of reducing feeding opportunities (Gilliam and Fraser 1987). For example, birds increase vigilance in the presence of a predator, thus decreasing the time spent foraging (Glük 1987). In marine habitats, predators may increase the use of vegetation cover by mobile invertebrates (Main 1987) and influence feeding behaviour (Skilleter and Peterson 1994) and survival (Irlandi et al. 1995) of bivalves. In the light of these considerations, next question could be: Are less complex habitats with food availability preferred by herbivores over more complex habitats without food availability? Then: How important is food type for seagrass herbivores choosing between seagrass habitats of equal complexity?

Do structurally complex seagrass patches provide better feeding conditions and refuge for mesograzers?

A primary reason why animal abundance scales with seagrass density or biomass is that they act as a physical and predation refuge. Habitat complexity, measured as shoot

density or biomass, can enhance epifaunal abundance via either providing protection from predators or selecting denser habitat to avoid physical stress of hydrodynamics (Boström and Mattila, 1999; Moore and Hovel, 2010; Schofield, 2003; this Thesis). Doing so is important because the two mechanisms imply very different patterns of trophic transfer to herbivores, on both local and global scales: if the higher mesograzer abundance in dense seagrass beds results solely from habitat selection to avoid physical stress, then trophic transfer to predators should be proportional to seagrass density. Conversely, if the relationship results solely from predation refuge, trophic transfer should be relatively invariant with seagrass density because epifauna are less vulnerable to predation in dense beds.

To deepen in the plant defense theory

Are there differences on tropical vs temperate defences in seagrasses?

Widespread literature suggests that plants from lower latitudes experience stronger biotic interactions (Pennings et al. 2007, Schemske et al. 2009) and therefore should invest more on chemical defenses (Coley and Aide 1991). A productive way for testing latitudinal relationships with herbivory rates in seagrass ecosystems would be to integrate plant functional traits approach (including physical and chemical defences) with hypotheses of past plant–herbivore interactions (e.g. see Vélez-Juarbe et al. 2012 about sirenians evolution). An integrated framework would better account for the costs of herbivory to a plant and better explain patterns of chemical defences across evolutionary and ecological scales.

What determine the synthesis of the compounds involved in deterring herbivores?

Current literature suggests that phenolic acids are common and widely distributed in seagrasses (Zapata and McMillan 1979). Phenolic acids usually found in seagrasses are classified within the group known as secondary metabolites. Rosmarinic acid is one of the most abundant phenolic compounds for *Zostera* genus, the most studied genus in the Atlantic region (e.g. Achamlale et al., 2009; Harrison, 1982; Manck et al., 2017; Vergeer et al., 1995; Vergeer and Develi, 1997; Zimmerman et al., 1995). Others secondary metabolites like caffeic acids and flavonoid glucosides such as quercetine and isorhamnetine monoglucosides have been also found in *Cymodocea nodosa* (Cariello et al. 1979, Sica et al. 1984). In this Thesis for the first time other secondary compounds for this temperate species have been recorded, after a previous acclimation to a future scenario of global change. Therefore, the production of phenolic acids requires the need for further research exploring whether local or global environmental conditions are drivers of any variation in the synthesis of natural products and the balance of their direct or indirect role in seagrass defence to herbivory.

Are diverse mesograzer assemblages more likely to influence seagrass resilience?

Throughout this Thesis we found that diversity (species richness and abundance) of macrophytes modifies feeding preference and then, positive consequences in seagrass resilience is expected (chapter 3). On the other hand, encouraging consequences in seagrass resilience have been showed in past studies by mesograzer diversity (Duffy et al. 2003, Best and Stachowicz 2012). Therefore, it will be exciting to conduct laboratory feeding assays to quantify per capita feeding rates on periphyton, macroalgae (e.g., *Ulva*), and seagrass by the dominant grazers, and test whether these rates are conserved by genus or family. The results could be used to estimate impacts expected in different communities as a function of measured mesograzer species composition and abundance.

Develop more comprehensive studies on seagrass response to herbivory damage

What effects have hydrodynamics on grazing responses in seagrass communities?

This Thesis has demonstrated that hydrodynamics influences the feeding and foraging behaviour of seagrass grazers, as well as in the leaf palatability of acclimated plants to different hydrodynamic regimes. Interestingly, new perspectives emerge on hydrodynamic effects and seagrass-grazer interactions based on *how currents can modulate plant response to herbivory damage*. Therefore, seagrasses inhabiting unsheltered sites will probably have greater resistance responses to the loss of biomass via grazing (perhaps indirectly to their environment adaptation) than those that inhabit in sheltered areas, which respond to herbivory through tolerance responses.

Does the type of mechanical damage derived from different bites affect plants at the physiological level?

While seagrasses compensate for leaf loss via the rapid recycling of stored nitrogen (Bjorndal 1980, Short and McRoy 1984, Pedersen and Borum 1993) and the transport of carbohydrates along common rhizomes (Libes and Boudouresque 1987, Tomasko and Dawes 1989), or grazing triggers increased nitrogen uptake from the surrounding environment by N requirements to reconstruct tissues (Jiménez-Ramos et al., in preparation b), a new hypothesis arises focusing on whether the type of leaf damage, derived from the type of bite (e.g. bitten *vs* ripped leaves), produces different internal damages triggering different physiological consequences (e.g., nitrogen translocation or enzymatic activity).

How herbivory damage influence seagrass community under warming temperatures?

Global warming is emerging as a major threat to ecosystems worldwide (Sala et al. 2000, IPCC 2007). Consequently, the effect of warming on seagrasses has been recently studied, since temperature is a key factor for seagrass health, growth and community

metabolic rates (Koch et al. 2013; Hernán et al. 2017). Recently, Egea et al. (2017) found a significant increase in the C metabolism and DOC fluxes of the community dominated by *C. nodosa* under a heat wave episode. On the other hand, others studies provided support for a mechanistic link between environmental temperature and feeding rates (Carr and Bruno 2013). In this thesis we have shown unpublished results where we demonstrate that the loss of half of the foliar biomass by grazing can lead to a shift from autotrophic to heterotrophic in a community dominated by *C. nodosa* conditions. Under these considerations, an interesting study would be the observation of how an *in situ* temperature increase would influence on the metabolism and DOC fluxes of the grazed seagrass community.

" I am constantly striving to understand the world around me. This internal drive inspires me to pursue a career in ecological research, specifically focusing on how the earth's changing climate and anthropogenic activities affect ecosystem services that humans rely on. I am interested in forecasting how these stressors will alter coastal ecosystems over time and determine what conservation measures can be taken to mediate and alleviate their impacts"

CONCLUSIONS

Ya que todo es el reflejo de nuestra mente, todo puede ser cambiado por nuestra mente.
Since everything is reflexion of pur minds, everything can be changed by our minds.

Buddha

Conclusions

1. Interactions among hydrodynamic conditions and habitat complexity (i.e. shoot density) significantly influenced the feeding and foraging behaviour of the amphipod *Gammarus locusta* in (artificial) seagrass meadows. These findings reinforce the importance of seagrasses as foundation species, having a positive biological effect on mesograzers through facilitation (i.e. reducing hydrodynamic pressure).

2. Hydrodynamics influence negatively annual defoliation rates in *Cymodocea nodosa* meadows. Exposed meadows showed lower biomass loss due to consumers than sheltered ones. This result highlights the role of hydrodynamics as a crucial factor in the seagrass-herbivore interactions, since it influences leaf traits and feeding capacity of consumers.

3. Seagrass acclimation to hydrodynamic conditions modulates intraspecific seagrass traits and has consequences in the selection of leaves independently of grazer species. Leaves with high nutritional quality showed higher defoliation rates than leaves with higher structural traits.

4. Macrophyte species diversity (i.e. species richness and abundance) may generate a preferential consumption over those macrophytes more palatable (i.e higher nutritional content and lower structural defenses) as is the case of *Ulva* sp., especially when *Paracentrotus lividus* has available large food abundance.

5. An increase of nutritional quality was recorded in *C. nodosa* leaves when subjected to nutrient enrichment. Therefore, under eutrophication higher palatability and consumption rates can be reached enhancing the negative effects recorded under such phenomenon.

6. The role of morphological, structural and biomechanical defences of *C. nodosa* leaves shifting their palatability, and thus affecting to their consumption rate by *P. lividus* has been demonstrated.

7. The presence of epiphytes in *C. nodosa* leaves significantly increased the consumption rate of seagrass leaves independently of their nutritional status.

8. The current main global drivers (eutrophication, acidification and warming) influenced positively *C. nodosa* palatability through changing in leaf traits. As a

consequence, *C. nodosa* plants acclimated to global change scenario were more consumed by *P. lividus*.

9. The leaf trait acclimation to global change scenario was highly variable among the three factors studied. This result reinforces the requirements of multi-scale approach from the level of individual responses to that of populations, communities and the entire ecosystem.

10. The colonization state of seagrass populations influences the compensatory responses in the tropical seagrass *Halodule wrightii* under simulated herbivory. Our findigings suggest that herbivores could have an important role in seagrass community structure and function, and could be a key force during succession dynamics.

Conclusiones

1. La interaccion entre las condiciones hidrodinámicas y la complejidad del hábitat (densidad de los haces) influyeron significativamente en el comportamiento alimenticio del anfípodo *Gammarus locusta* en praderas marinas (artificiales). Estos resultados refuerzan la importancia de las angiospermas marinas como especies fundadoras, las cuales tienen un efecto biológico positivo sobre sus consumidores facilitando su consumo y movilidad.

2. La hidrodinámica influye negativamente en las tasas anuales de defoliación en las praderas de *Cymodocea nodosa*. Aquellas praderas altamente expuestas al régimen hidrodinámico mostraron menos pérdida de biomasa por consumo que las praderas que habitan en zonas protegidas. Este resultado muestra como la hidrodinámica es un factor crucial para la comprensión de los procesos de herbivoría en comunidades de angiospermas marinas, ya que influye tanto en las propiedades de las hojas y la capacidad de alimentación de los consumidores.

3. La adaptación de las angiospermas marinas a las condiciones hidrodinámicas da lugar a una variación de las propiedades foliares dando lugar a diferencias intraspecíficas. Estas diferencias intraespecífcas conllevan una diferenciación en la selección de hojas por parte de los consumidores. Las hojas con alta calidad nutricional mostraron tasas de defoliación mayores que las hojas con altos rasgos estructurales.

4. La presencia y abundancia de diferentes especies de macrófitos puede generar un consumo preferencial sobre el macrófito más palatable (es decir, aquel con alto contenido nutricional y bajas defensas estructurales). El erizo de mar *Paracentrotus lividus* mostró un mayor consumo sobre *Ulva* sp. frente a las angiospermas marinas *Cymodocea nodosa* y *Zostera noltei*, especialmente bajo condiciones de alta abundancia de alimento.

5. Respecto a los efectos de la eutrofización, se registró un aumento de la calidad nutricional, mejorando la palatabilidad de *Cymodocea nodosa*. Esto se debió principalmente al aumento del contenido en N de las hojas.

6. La función de las defensas morfológicas, estructurales y biomecánicas de las hojas de *Cymodocea nodosa* frente al consumo de *Paracentortus lividus* ha sido demostrado.

7. La presencia de algas epífitas en hojas de *Cymodocea nodosa* aumentó significativamente su consumo por parte de *Paracentortus lividus*, debido al incremento de su calidad nutricional.

8. Los principales factores relacionados con el cambio global (eutrofización, acidificación y calentamiento) influyeron positivamente en la palatabilidad de *Cymodocea nodosa* a través de la variabilidad en las propiedades de las hojas. Como consecuencia, los haces aclimatados a diferentes condiciones de cambio global fueron más consumidos.

9. Las respuestas de las propiedades foliares a diferentes condiciones de cambio global fueron altamente variables. Este resultado refuerza los requisitos de un enfoque a multi-escala desde un nivel de respuestas individuales a respuestas de poblaciones, comunidades y todo el ecosistema.

10. Las etapas de recuperación influyeron en las respuestas compensatorias a la pérdida de hojas por herbivoría en la angiosperma marina tropical *Halodule wrightii*. Nuestros hallazgos sugieren que los herbívoros podrían tener un papel importante en la estructura y función de la comunidad de angiospermas marinas, y podrían ser clave durante la dinámica de sucesión de parches.

Conclusions

1. La interacció entre les condicions hidrodinàmiques i la complexitat de l'hàbitat (densitat de fulles) van influir significativament en el comportament alimentari del amfípode *Gammarus locusta* en praderies marines (artificials). Aquests resultats reforcen la importància de les angiospermes marines com a espècies fundadores, les quals tenen un efecte biològic positiu sobre els seus consumidors facilitant el seu consum i mobilitat.

2. La hidrodinàmica influeix negativament en les taxes anuals de defoliació a les praderies de *Cymodocea nodosa*. Aquelles praderies altament exposades al règim hidrodinàmic van mostrar menys pèrdua de biomassa per consum que les praderies que habiten en zones protegides. Aquest resultat mostra com la hidrodinàmica és un factor crucial per a la comprensió dels processos d'herbivorisme en comunitats d'angiospermes marines, ja que influeix tant en les propietats de les fulles i la capacitat d'alimentació dels consumidors.

3. L'adaptació de les angiospermes marines a les condicions hidrodinàmiques dóna lloc a una variació de les propietats foliars i com a conseqüència, a diferències intraspecífiques. Aquestes diferències intraespecífcas comporten una diferenciació en la selecció de fulles per part dels consumidors. Les fulles amb alta qualitat nutricional van mostrar taxes de defoliació més grans que les fulles amb altes propietats estructurals.

4. La presència i abundància de diferents espècies de macròfits pot generar un consum preferencial sobre el macròfit més palatable (és a dir, aquell amb alt contingut nutricional i baixes defenses estructurals). L'eriçó de mar *Paracentrotus lividus* va mostrar un major consum sobre *Ulva* sp. enfront de les angiospermes marines *Cymodocea nodosa* i *Zostera noltei*, especialment sota condicions d'alta abundància d'aliment.

5. Pel que fa als efectes de l'eutrofització, es va registrar un augment de la qualitat nutricional, millorant la palatabilitat de *Cymodocea nodosa*. Això es va deure principalment a l'augment del contingut en N de les fulles.

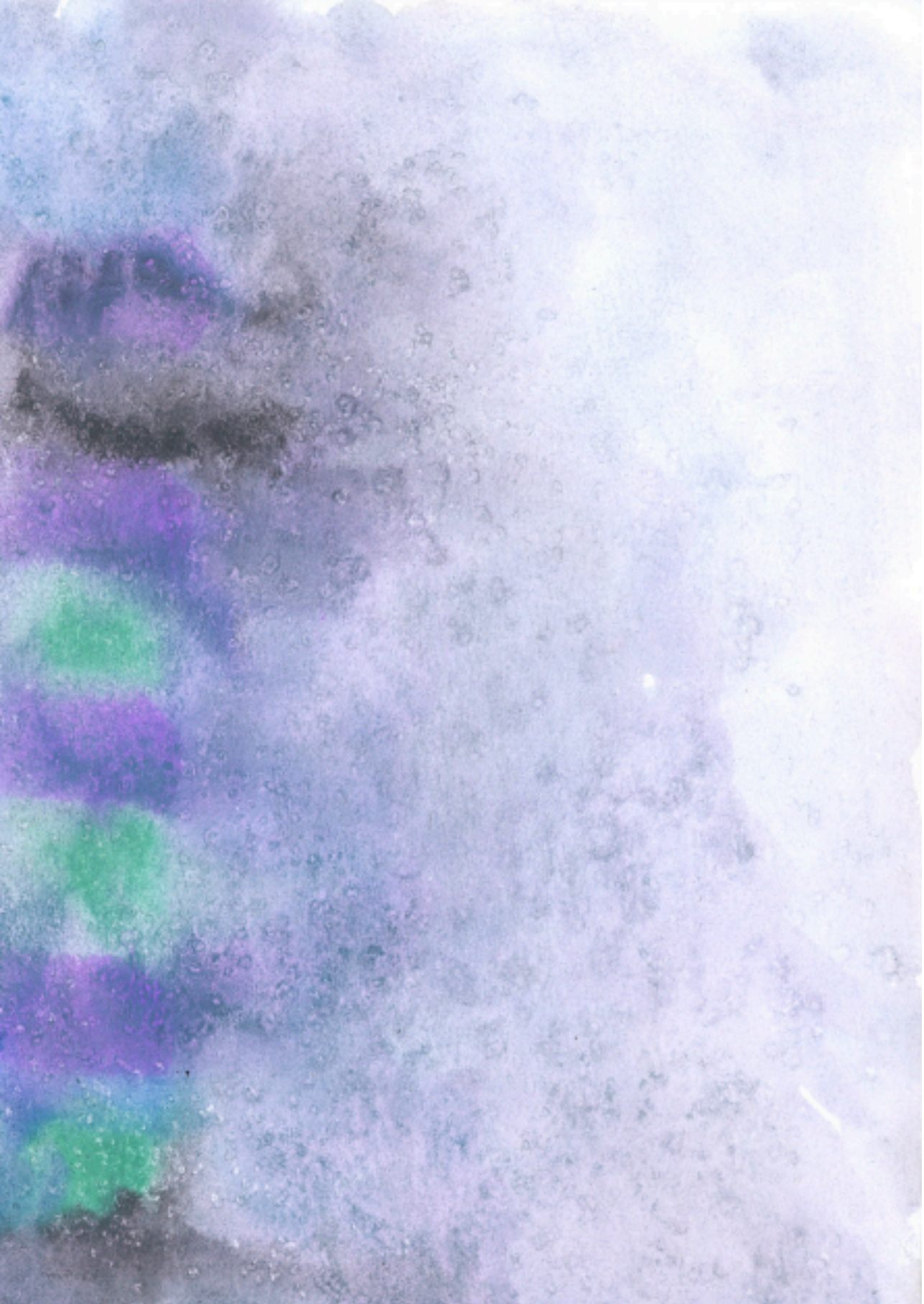
6. La funció de les defenses morfològiques, estructurals i biomecàniques de les fulles de *Cymodocea nodosa* enfront del consum de *Paracentortus lividus* ha estat demostrat.

7. La presència d'algues epífites en fulles de *Cymodocea nodosa* va augmentar significativament el seu consum per part de *Paracentortus lividus*, a causa del increment de la seva qualitat nutricional.

8. Els principals factors relacionats amb el canvi global (eutrofització, acidificació i escalfament) van influir positivament en la palatabilitat de *Cymodocea nodosa* a través de la variabilitat en les propietats de les fulles. Com a conseqüència, les fulles aclimatades sota diferents condicions de canvi global van ser més consumides.

9. Les respostes de les propietats foliars a diferents condicions de canvi global van ser altament variables. Aquest resultat reforça els requisits d'un enfocament a multi-escala des d'un nivell de respostes individuals a respostes de poblacions, comunitats i tot l'ecosistema.

10. Les etapes de recuperació van influir en les respostes compensatòries a la pèrdua de biomassa per l'herbivorisme a l'angiosperma marina tropical *Halodule wrightii*. Les nostres troballes suggereixen que els herbívors podrien tenir un paper important en l'estructura i funció de la comunitat d'angiospermes marines, i podrien ser clau durant la dinàmica de successió de praderies.



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La historia de la humanidad es básicamente una lista de cosas que no se podía hacer y luego se hicieron.
The history of humanity is basically a list of things that could not be done and then made..

Boyan Balt

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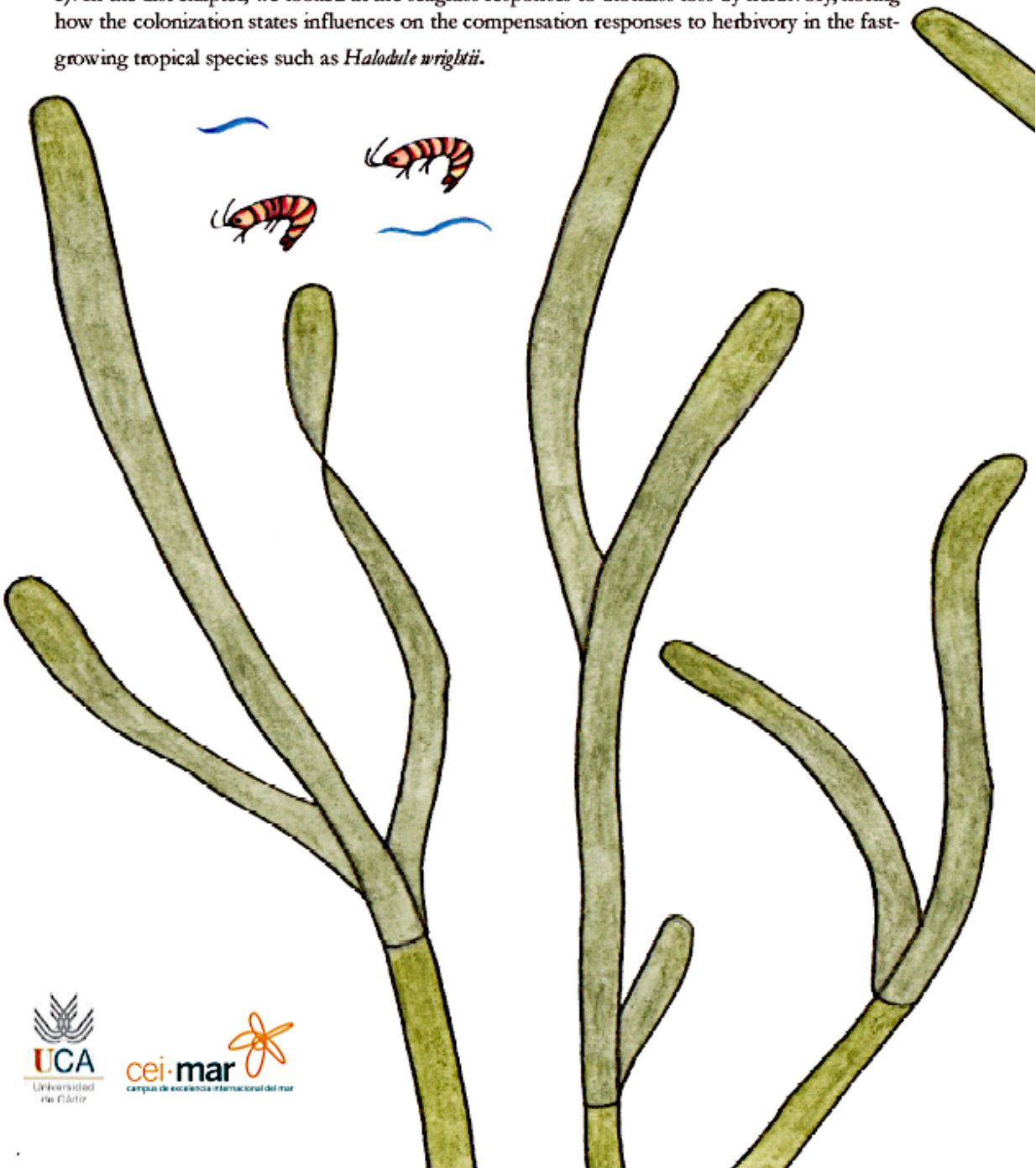
The PhD Thesis is structured in 6 chapters that posed particular questions about seagrass-grazer interactions. In the first two chapters, the importance of hydrodynamics in the seagrass-grazer interactions was studied, and it showed how this environmental forcing acts as a top-down regulator in the community. Moreover, seagrass acclimation to hydrodynamics modulates intraspecific seagrass traits, and then seagrass palatability, influencing the selection of leaves by consumers (chapters 2 and 3). On the other hand, eutrophication and the main global change factors (i.e. warming and acidification) influenced positively *Cymodocea nodosa* palatability through the alteration of leaf traits and by promoting the growth of epiphytes (chapters 4 and 5). In the last chapter, we looked at the seagrass responses to biomass loss by herbivory, noting how the colonization states influences on the compensation responses to herbivory in the fast-growing tropical species such as *Halodule wrightii*.



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